(Abstract of the thesis for Ph.D. degree to be submitted by S.C. Gupta.)

"The Production and Properties of Pectolytic and Macerating Enzymes Secreted by Pythium deBaryanum Hesse."

The work presented is on the pectolytic enzyme production of the fungus Pythium deBaryanum when grown in artificial culture. In addition to confirming and amplifying the results of earlier workers, it has been shown that sodium chloride and caramelized glucose markedly stimulate enzyme production. The caramelization of glucose takes place in a medium when the glucose is autoclaved in presence of the potassium phosphate (tribasic) component of the medium. Caramelization with a similar stimulating effect may also be brought about by heating dry glucose at 155°C. Chromatographic analysis of caramelized glucose solution revealed that a mixture of glucose, mannose, fructose, dihydroxyacetone, and glyceraldehyde is formed during the process. It has been suggested on experimental results that glyceraldehyde, either alone or with some sugars, is the main cause of the stimulating effect shown by caramelized glucose.

It has been demonstrated that P. deBaryanum differs from Botrytis cinerea in that the former does not secrete polygalacturonase and pectin-esterase. There is evidence of a 'depolymerase' enzyme secreted by P. deBaryanum. The effect of pH, of purification, and of temperature on the activity of pectolytic enzymes secreted by P. deBaryanum has been studied and compared with the corresponding behaviour of B. cinerea and Bacterium aroideae enzymes.

The conclusions are reached that protopectinase and polygalacturonase are two different enzymes and that there is evidence for another enzyme 'depolymerase'.

THE PRODUCTION AND PROPERTIES OF PECTOLYTIC AND MACERATING ENZYMES SECRETED BY PYTHIUM DEBARYANUM HESSE.

A Thesis presented by

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I INTRODUCTION

Studies in the Physiology of Parasitism, which have been carried out in the Plant Pathological Laboratory of Imperial College, have been directed, among otherthings, to an examination of the enzymic properties of pathogenic fungi. Certain parasites such as Botrytis cinerea or Bacterium aroideae produce pectolytic enzymes on a great variety of media and over a wide range of conditions. With Pythium deBaryanum, however, it appeared to be otherwise. It was clear that this fungus readily produced a macerating type of enzyme when growing on a living host, but on most of the usual culture media, natural or synthetic, little enzyme was produced. In due course it was found that good enzyme preparation could be obtained on certain media, when the ingredients and proportions were suitably chosen. It was also found that the method of preparing the medium considerably affected its value from the point of view of enzyme production. The further examination of this problem, viz. the rather specialised behaviour of P.deBaryanum in the matter of pectolytic enzyme secretion together with the study of the properties of these enzymes, is the topic of this paper.

First Part. FACTORS AFFECTING ENZYME PRODUCTION IN CULTURAL MEDIA

A. HISTORICAL ACCOUNT.

Various workers from time to time have noted that the culture medium, whether natural or synthetic, has a marked effect on the production of pectinase* by various micro-organisms. The technique for preparation of such an enzyme was worked out by Brown (1915, 1917), who germinated large quantities of spores of Botrytis cinerea on horizontal glass plates and obtained the enzyme, either by grinding and extracting the germinated spores (endo-enzyme), or by use of the liquid in which germination had taken place (exoenzyme). He also observed that the activity of endo- and exo- enzyme preparations was dependent on (1) the density of sowing of spores, (2) the time allowed for germination and growth, and (3) the nature of nutrient medium. A thick sowing of the spores and 1-2 days' incubation period was found to be more suitable for obtaining endoenzyme, while 4-6 days' incubation was required to get the most active preparations of exoenzyme.

A large variety of media, such as potato extract, turnip

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* Throughout the First Part, the term 'pectinase' enzyme is applied to an enzyme responsible for the hydrolysis of the protopectin of the middle lamella of cells and for the consequen loss of coherence of the tissue. The full terminology of this and other enzymes is described in Second Part.

extract, grape juice, proved to be quite satisfactory for Botrytis cinerea.

Harter and Weimer (1921, 1923) working with Rhizopus tritici obtained exoenzyme on various natural media though not on prune juice. They failed to obtain an active enzyme preparation on a synthetic solution where glucose was the source of carbon. When glucose was replaced by pectin in Czapek's solution, an active enzyme was secreted by the fungus.

In a comparative study of the pectinase enzymes of Botrytis cinerea, Fusarium fructigenum, Phytophthora erythroseptica, and Pythium de-Baryanum, Chona (1932) found that Brown's technique was not practicable with the latter two fungi as spores were not freely available. He modified the method by preparing culture solutions in flasks and inoculating them with pieces of fungal mycelium. For liquid cultures, the juice from potato slices was extracted by means of a hand press and used as such. No enzyme, either in the mycelium or in the culture medium, could be demonstrated by this method. However, he was able to get active preparations of enzyme by inoculating large, peeled potato tubers after surface sterilization with a 1:1000 mercuric-chloride solution and squeezing the rotted tissue after 6 days' incubation at 20°C.

Menon (1934) failed to obtain active preparations of enzyme from Pythium de-Baryanum when it was allowed to grow on a variety of media such as minced potato extract, potato mush, turnip extract, oatmeal extract, Richards' Solution, and Brown's Synthetic Medium. He reported difficulty in obtaining

enzyme, from potato tubers by the method described by Chona on account of bacterial contamination. Later he solved this problem by using either autoclaved potato tubers or sterilized plugs of potato tissues and then extracting the sap from the rotted portions.

Fernando (1937) in a further enzymological study of Pythium de-Baryanum used Menon's method and found it to be quite satisfactory.

The first detailed attempt to find a suitable culture solution for enzyme secretion by Pythium de-Baryanum was made by Ashour (1949). He found potato decoction and minced potato extract to be quite suitable for the purpose. In the case of potato decoction (200 gms. in 1 litre of distilled water), he observed that activity of enzyme secretion increased with dilution upto about 1 in 5. He was unable to get an active enzyme preparation on turnip decoction.

Ashour also studied enzyme secretion on various synthetic media. In his preliminary work, he used Brown's Medium A, Brown's Medium B, Richards', Glucose-peptone, and modified Czapek's medium, but found that only the first was suitable for enzyme secretion. Using as a basis the analysis of dried potato as given in Thorpe's Dictionary of Chemistry, Ashour modified Brown's Medium A into a 'Standard Synthetic Medium' which contained 2.5% starch, 0.5% glucose, 0.45% peptone, 0.45% asparagine, 0.34% K_3PO_4 and 0.19% $MgSO_4.7H_2O$. This 'standard' medium was the most suitable for the secretion of active enzyme by the fungus. He found that pectin was a very

poor source of carbohydrate for the growth of Pythium de-Baryanum, and that in fact it was harmful in a medium otherwise suitable for enzyme production.

Damle (1952) was not able to obtain active preparation of the enzyme when Pythium de-Baryanum was grown on Ashour's 'Standard Synthetic Medium'. In preparing the medium he autoclaved the phosphate separately from the other constituents in order to minimise precipitation of magnesium phosphate. Ashour does not state specifically how he carried out autoclaving but it is presumed that all the constituents were mixed together in advance. It was only when the carbohydrate part was autoclaved with the phosphate that Damle obtained a medium which gave strong pectinase activity. He suggested that small quantities of certain phosphoric esters of glucose and fructose are formed by heating carbohydrates with phosphate. These phosphoric esters were supposed to be responsible for the stimulation of enzyme secretion.

Incidentally, Damle noted that when sugar was autoclaved with alkaline phosphate (K_3PO_4) a brown colour developed, which did not appear when the sugar was autoclaved with any or all of the other constituents. Therefore it came about that the medium did not give enzymic activity unless it showed this brown colour.

The effect of heating together phosphate and sugar on the growth of filamentous fungi has not hitherto been reported. But there are a few references concerning a similar phenomenon

in studies of bacteria and yeasts. Thus Bulmer and Huesselmann (1927) state that when glucose and K_2HPO_4 are autoclaved under pressure, substances are formed which stimulate the growth of yeast. Later Bulmer, Williams, and Werkman (1931) report that under these conditions bacterial growth substances are formed. The darkening effect was interpreted to mean that some caramelization had taken place but tests with commercial caramel of unknown origin and with glucose which had been caramelized (by heating at 200°C for 20 minutes) gave only slight stimulation. Conversely when caramelized medium was decolourised by active charcoal, it retained the stimulating power. They concluded that the stimulation was not due to caramelization as such but to some substance produced at the same time as the caramel.

Lewis (1930) found that Phytomonas malvacearum fails to grow in culture medium containing reducing sugars (glucose, maltose, lactose, galactose, Laevulose) nitrogen and K_2HPO_4, when sterilized at 122°C for fifteen minutes. This inhibition of growth was not observed when sucrose or glycerol was used, or when the medium was sterilized at lower temperatures, or by filtration. When the medium was adjusted to pH 5.4 before sterilization, no colour or inhibition was observed. Heating at an alkaline pH but in absence of phosphate caused no inhibition. He concluded that it was an interaction between phosphate, glucose and a nitrogenous compound which gave rise to an inhibitory substance.

A similar effect is described by Baumgartner (1938) who

found that substances toxic to Escherichia coli were produced by some methods adopted in sterlizing alkaline media containing reducing sugars. Media which were toxic, were dark in colour but it was shown by decolourizing with charcoal that the dark substance was not the active agent (compare Fulmer, ~~and~~ & Werkman Williams above).

The present investigation was undertaken to study the effect of the composition of synthetic media and the method of sterilizing these media on the secretion of pectic enzymes by Pythium de-Baryanum.

B. GENERAL MATERIALS AND METHODS.

The fungus used in this work was an isolate of Pythium de-Baryanum Hesse, obtained from the stock cultures of the Plant Pathology Laboratory, Imperial College of Science and Technology, London. The fungus was maintained on potato dextrose agar (20% peeled potato, 2% dextrose and 2.0% agar). After inoculation cultures were incubated for three days at 25°C and were then kept at room temperature. Fresh cultures were prepared at monthly intervals.

As media for the preparation of pectinase enzyme, both natural and synthetic solutions were used, the latter especially. Natural solutions were all prepared from potato tubers. A variety of methods was used, whereby solutions of somewhat different strength and composition were obtained. The details will be given in section C.2. below.

The basis of the synthetic medium was Ashour's formula, viz.-

25.0 grms. 'Analar', starch (Hopkin & Williams Ltd.)
5.0 grms. Glucose. (" " " ")
4.5 grms. Peptone (Bacteriological, G.T. Gurr Ltd.)
4.5 grms. Asparagine (Hopkin & Williams Ltd. or Solmedia Ltd.)
3.4 grms. K_3PO_4
1.9 grms. $MgSO_4.7H_2O$.

made up to 1 litre with distilled water.

In conformity with the text of Damle's paper (l.c.) this

medium will be referred to as 'S' (for 'standard'). Later in the work an improved form of this medium was devised, the improvement being that it was easier to prepare and it gave more active enzymic preparations. This medium, which will be referred to as M (for modified) is as follows:

30 grms. Glucose
4.5 grms. Peptone
4.5 grms. Asparagine
3.4 grms. K_3PO_4
1.9 grms. $MgSO_4 . H_2O$
1.0 grms. NaCl

made up to 1 litre with distilled water.

For preparation of enzyme solutions 15 mls. of the medium were placed in flat medecine bottles of 12 fluid oz. capacity. Throughout the work special care was taken to wash these bottles thoroughly before use. They were sterilized by heating at 15 lbs. pressure for 20 minutes.

Cultures for inocula were grown for 2-3 days at 25°C. in Petri dishes containing 0.2% glucose agar. Uniform inocula were cut from the growing edge of these cultures by means of a sterilized No.1 cork borer (4 mm. diam.). Ten such inocula were placed in each culture bottle. The latter, were stacked on their sides and incubated at 25°C for 5 days.

At the end of the incubation period the fungal mats were removed, washed in running hot water, dried on tared filter papers at 70-80°C and weighed on a Joly Spring balance.

Dry weights of the mycelium are given in Joly units (15 Joly unit = 1.0 grms). The filtrate from the bottles was tested as such for the activity of enzyme by the potato disc method of Brown (1915).

Cylinders of potato tubers (var. King Edward) were cut with No. 5 cork borer (9 mm. diam.) and made fully turgid by injecting with water under the air pump. They were then cut into discs 0.5 mm. thick using a hand microtome. These discs were washed with distilled water, partially dried on filter paper and three were placed in 3 mls. of an enzyme preparation in a watch glass. Maceration of the discs was estimated by determining the time required for loss of coherence. This time (R.T.) is an inverse measure of enzyme activity.

A crude enzyme solution was also prepared by infecting surface sterilized healthy potato tubers by the method described by Granger & Horne (1924) and extracting the sap from the rotted tissues.

When required enzyme preparations were kept for long periods in small bottles at -20°C. For periods of not more than one week, preparations were kept at 4°C. under toluene, without appreciable loss of activity.

pH changes were followed either colorimetrically or by use of a pH-meter, or by both methods, according to the needs of the particular experiment.

C. EXPERIMENTAL RESULTS.

1. Preliminary Examination of Synthetic Medium

The work was started according to the method described by Damle (l.c.). Each bottle contained finally 25 ml. of standard synthetic medium 'S', the magnesium sulphate being autoclaved separately from the rest of the medium. Each bottle was inoculated with six discs from a two-day culture of Pythium de-Baryanum. Bottles were then incubated at 25°C for five days and the liquid after this period was tested for the activity of enzyme.

The results obtained were disappointing as the macerating time (R.T.) for potato discs of standard thickness was found to be about 120 minutes as against the 30-35 minutes observed by Damle. It was thought that the strain, which was obtained from the stock cultures might have lost in virulence. To ensure its pathogenicity it was passed through potato tubers (King Edward var.) and then reisolated. The fresh isolate, however, was only slightly more active in enzyme production (R.T. 110 minutes).

In further attempts, the quantity of culture medium was reduced from 25 to 10 mls. and the inoculum was increased from 6 to 10 culture discs in each bottle. This variation in the method resulted in more active enzyme production bringing the maceration time (R.T.) to 55-60 minutes. Further increase in inoculum per bottle and variation in incubation period did not improve the yield of enzyme.

In view of these failures to obtain sufficiently active enzymic solutions, it was decided to carry out a detailed study of enzymic secretion on various types of potato media and on various modifications of the synthetic medium.

2. Enzyme Secretion on Natural Media.

Tubers of the variety King Edward, were inoculated after surface sterilization with 95% alcohol. A cavity 0.25 mm. deep was made in the tuber with a sterilized No.2. cork borer (6mm. diam.), plugged with a disc of culture and sealed with paraffin wax. Tubers so inoculated were incubated at 25°C for 7-10 days in tin boxes (10" x 10"). The rotted portions of tubers were then collected by scraping out with a glass spatula and pressing through muslin to give a crude enzyme preparation which was then centrifuged to get rid of starch and other debris.

At the same time the fungus was grown in liquid cultures (25 ml. per bottle) on three types of potato extract, as follows:

P_1 - 200 grms. potato tuber boiled for 45 minutes in distilled water, filtered through fine muslin and the filtrate made up to 1 litre.

P_2 - Potato tuber finely minced, squeezed through muslin and centrifuged. The clear extract was then used.

P_3 - Potato tubers frozen at -20°C for 24 hours, then brought to room temperature by placing in running tap water; sap squeezed out by hand and cleared by centrifuging.

The precipitate which formed on sterilizing media P_2 and P_3 was left in the medium. The activity of secreted enzyme (after 5 days' growth) is shown in Table I.

Table I

Macerating Activity on various Potato Media.

Medium	Initial pH	Final pH	R.T. in mins.
Living potato tuber	-	7.0	25-30
P_1	6.0	7.8	20-25
P_2	6.0	8.2	35-40
P_3	5.9	8.2	25

Active enzyme preparations were obtained on all these natural media.

Table II gives the activity of pectinase preparations on various dilutions of media P_1, P_2 and P_3. The effect of the precipitate formed when P_2 and P_3 are autoclaved was also determined.

Table II

Enzyme Production on Various Preparations of Potato Extracts.

Medium	Initial pH	Final pH	R.T. in mins.	Dry wt. of Myce.
P_1	6.0	7.8	22	0.85
$P_{1/2}$	6.1	7.8	35	0.5
$P_{1/4}$	6.2	8.0	40	0.25
P_2 with ppt.	6.0	8.2	40	-
" without "	6.0	8.4	60	0.3
$P_{2/2}$ with "	6.1	8.5	60	-
" without "	6.3	8.7	90	0.2
P_3 with "	5.9	8.2	25	1.05
" without "	5.9	8.1	25	1.55
$P_{3/2}$ with "	6.0	8.5	25	0.65
" without "	6.0	8.4	25	0.8
$P_{3/4}$ with "	6.2	8.6	30	0.3
" without "	6.2	8.5	30	0.45

On all the media, the pH became alkaline, and more rapidly so the more dilute the medium. Media P_1 and P_3 gave active extracts, P_3 being best in as much as it could be diluted to quarter-strength without appreciable effect on its capacity to produce pectinase. P_2, though it was like P_3 in being an undiluted extract of potato, was distinctly poorer for enzyme production, a result which is also shown

in Table I and in a second experiment along the lines of that described in Table II. The reason for this difference is possibly that the mincing of tissue involved in the preparation of P_2 speeds up certain oxidation reactions; recent work by Cole (1953) has shown that certain oxidised plant juices have a greater retarding action on pectinase activity than similar juices in which oxidation was prevented.

The presence or absence of the precipitate had no obvious effect on P_3 media with respect to enzyme production; with P_2 media there was an effect, less enzymic activity being shown when the precipitate was removed.

The dry weight of mycelium, as was to be expected, was reduced in dilutions of the media. With each particular extract (viz. P_2 and P_3) there was an inverse correlation between dilution and enzymic activity, but this correlation broke down when P_1 media were compared with P_3 media.

Synthetic medium 'S' is presumed to contain all the essential components of the potato juice, but in the experiments so far recorded it was definitely inferior to potato media. The possibilities therefore seemed to be:-

(1) Production of pectinase enzymes by P.de-Baryanum is to some extent adaptive, being intensified by the presence of pectic substances in the medium.

(2) Some growth substances, which are present in the natural medium, are required.

(3) Some salt present in potato tissue is missing from the synthetic medium.

An examination of these factors will be given in the section following.

3. Detailed Study of Enzyme Secretion on Synthetic Medium

(a) Effect of Pectic Substances.

The effect of adding two types of pectic substances is shown in Table III. One of them was a preparation of 'Citrus pectin' (Methoxyl groups 10.5%) and the other 'Sodium polypectate' (ammonium 1.3%, sodium 4.2%) both supplied by California Fruit Growers Exchange. In all medis of Table III the magnesium sulphate was autoclaved separately from the remainder of the medium.

Table III

Effect of Pectic Substances on Enzyme Production

Medium	Initial pH	Final pH	R.T. in Mins.	Dry wt. of Myce.
S	8.0	8.0	90	2.0
S with 1% pectin	5.9	7.5	30	2.2
S with 1% sodium polypectate	6.4	7.6	30	2.7

The addition of pectic substances somewhat increased mycelial growth and gave distinctly more active enzymic preparations. The suggestion is that the enzyme production was stimulated by the presence of pectic substances. The effect, however, was complicated by the possible presence of certain ions, as will be shown later (3, c).

(b) Effect of Growth Substances.

'Yeastrel' a preparation rich in various salts, certain amino-acids and growth substances (supplied by Brewer's Food Supply Co. Ltd., Edinburgh) was added in various concentrations to synthetic medium 'S'. The results are shown in Table IV.

Table IV.

Effect of 'Yeastrel',on Enzymic Production

Medium	Initial pH	Final pH	R.T. in Mins.	Dry. Wt. of Myce.
S	8.0	8.2	110	2.3
S + 0.05% Yeastrel	"	"	25	2.05
" + 0.1% "	7.9	"	"	2.15
" + 0.25% "	"	"	"	-
" + 0.5% "	7.8	"	"	-
" + 1.0% "	7.6	"	30	2.25
" + 2.5% "	7.2	8.1	40	-
" + 5.0% "	6.6	8.0	"	2.8

The above experiment showed that 'Yeastrel', had a definite stimulating effect on the secretion of active enzyme by the fungus. Higher doses gave more fungal growth but less active enzyme.

In view of the above results, the addition to medium 'S' of biotin, aneurin, nicotinamide and riboflavine was tested. Each of these substances was used at a concentration of 30γ%.

There was no effect on enzyme production as is shown in Table V.

Table V.

Effect of Vitamins on Enzyme Production.

Medium	Initial pH	Final pH	R.T. in Mins.
S	7.9	7.9	80-90
S + biotin	"	8.0	"
" + aneurin	"	7.9	"
" + nicotinamide	"	"	"
" + riboflavine	"	"	"

(c) Effect of Salts.

Solutions of ferric chloride, ferrous sulphate, calcium carbonate, calcium nitrate, calcium chloride, sodium chloride and sodium silicate were added to medium 'S' before sterilization. The ferric chloride and ferrous sulphate were used at 0.01% concentration, the others at 0.1% . The results are shown in Table VI.

Table VI.

Effect of Iron, Calcium and Sodium Salts on Enzyme Secretion.

Medium	Initial pH	Final pH	R.T. in Mins.	Dry. wt. of Myce.
S	8.0	8.0	110	2.0
S + $FeCl_3$	7.9	"	40	2.2
" + $Fe_2(SO_4)_3$	8.0	7.8	140	1.8
" + $CaCO_3$	"	8.0	90	1.3
" + $Ca(NO_3)_2$	7.9	7.7	140	1.1
" + $CaCl_2$	7.8	7.9	130	1.45
" + NaCl	8.0	8.2	25	2.1
" + Na_2SiO_3	"	"	120	2.1

The following conclusions may be drawn from the above experiment.

1. Ferric chloride stimulates enzyme production while addition of ferrous sulphate in medium 'S' results in poor activity of secreted enzyme.
2. Calcium nitrate and calcium chloride definitely retard enzyme production while calcium carbonate slightly stimulates.
3. Sodium chloride has a marked stimulating effect on enzyme secretion while sodium silicate retards.
4. Poor growth of mycelium results when calcium salts are added in the medium.

The striking effect of sodium chloride on secretion of enzyme by the fungus is confirmed by the results shown in Table VII which is based upon twelve separate experiments.

Table VII

Effect of 0.1% NaCl on Enzyme Secretion.

Medium	R.T. (in Minutes) of Experiments											
	1	2	3	4	5	6	7	8	9	10	11	12
S	90	70	180	60	80	110	50	45	70	65	50	90
S + NaCl	25	25	30	20	22	25	20	20	20	25	25	30

In view of the above findings a range of concentrations of sodium chloride was added in medium 'S' to find the optimum quantity of this salt required for enzyme secretion. The results are shown in Table VIII.

Table VIII

Effect of Various Concentrations of NaCl on Enzyme Secretion

Medium	Initial pH	Final pH	R.T. in Mins.
S	8.0	8.0	90
S + 0.001% NaCl	"	"	40
S + 0.0025% "	"	"	"
S + 0.005% "	"	8.1	35
S + 0.01% "	"	"	30
S + 0.025% "	"	"	25
S + 0.05% "	"	"	"
S + 0.1% "	"	"	"
S + 0.25% "	"	"	30
S + 0.5% "	"	8.2	40

(Magnesium sulphate, Sodium chloride and Potassium phosphate used in this experiment were 'Analar' chemicals.)

From the above experiment it is clear that the presence of sodium chloride in medium 'S', even at 0.001% concentration produced a stimulating effect on enzyme secretion. The most suitable concentration lay between 0.025% and 0.1%.

The effect of sodium chloride on enzyme secretion was so marked that another set of experiments was set up to find out whether the Na^+ or the Cl^- was the more important. The results are shown in Table IX.

Table IX.

Effect of Na^+ and Cl^- ions on Enzyme Production

Medium	Initial pH	Final pH	R.T. in Mins.
S	8.0	8.0	70
S + 0.1% $CaCl_2$	7.7-7.8	7.9	100
" + " NH_4Cl	7.8	7.7	25
" + " $MgCl_2$	7.9-8.0	8.0	"
" + 0.05% HCl	7.7	8.1-8.2	"
" + 0.1% KCl	7.9-8.0	"	23
" + " NaCl	8.0	8.2	"
" + 0.05% NaOH	8.3	8.2-8.3	30
" + 0.1% $NaNO_3$	8.0	8.0	50
" + 0.05% Na_2CO_3	8.3	8.2-8.3	"
" + " Na_3PO_4	"	8.3	40

From these results it is clear that all substances tested, which markedly stimulated enzyme secretion, contained either Na^+ or Cl^- and that the presence of one of these is therefore necessary for active enzyme production. The presence of Ca^{++} on the other hand is inhibitory and more than counteracts the stimulating action of the associated Cl^-.

The stimulating effect of sodium chloride on enzyme secretion was also confirmed by the viscometric method.

For each determination 5 mls of 1.0% washed citrus pectin* solution was adjusted to pH 8.3 with 0.1 N NaOH, 1 ml of Sorensen's phosphate buffer added to maintain pH at this point, and the volume brought to 8 mls by addition of distilled water. The pectin solution was then warmed to 25°C in a water bath and 2 mls of enzyme added. The mixture was stirred and 5 mls of it transfered to an Ostwald viscometer in a water bath at 25°C. The first viscosity determination was made one minute after adding the enzyme and then at intervals over a period of 30 minutes.

In Fig. 1, curves A and B give a measure of the activities of the respective enzymic preparations from cultures on media S and on the same with 0.1% NaCl added. In the former a viscosity of 51.8 sec. is reached in 30 minutes, in the latter the same viscosity reached in 2 minutes, indicating a fifteenfold increase in activity (see Second Part Sec. C, 2b.).

(d) Effect of Source and Concentration of Carbohydrates.

The presence of starch in medium S is inconvenient and is a likely cause of irregularity. Experiments were therefore carried out to see how far it could be replaced by various sugars.

Table X summarises the results obtained when starch is replaced, partially or wholly, by glucose.

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* Pectin was washed with 80% ethyl alcohol acidified with hydrochloric acid, as described by Kertesz (1951, p.122).

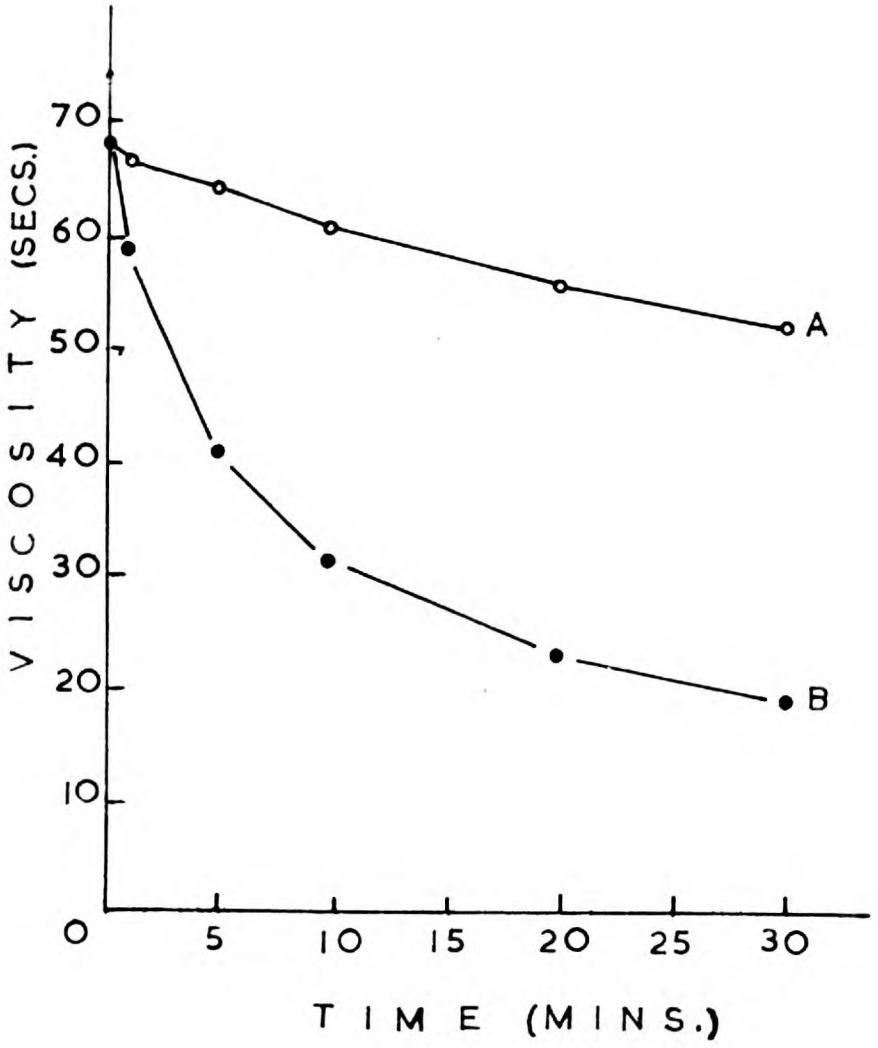


Fig. 1. Effect of sodium chloride on pectolytic enzyme production by P.deBaryanum.

A - Medium S; B - medium S + 0.1% NaCl.

Table X

Replacement of Starch with Glucose

Medium * with	Init. pH	Final pH	R.F. in Mins.	Dry. wt. of Myce.
3.0% Starch	8.1	7.8	180	1.7
2.5% " + 0.5% Glucose	8.0	8.1	25	1.9
1.5% " + 1.5% "	7.8	8.2	22	2.1
0.5% " + 2.5% "	7.6	8.2	22	2.2
3.0% Glucose	7.5	8.2	22	"

* Medium S with 0.1% NaCl.

Replacement of starch with glucose gave a medium more suitable for enzyme secretion. Starch, without glucose, gave low enzyme secretion and a somewhat decreased growth of mycelium. In the presence of glucose, a yellowish colour appeared on sterilization and with increasing glucose content this colour became brown.

The lower pH values shown in the autoclaved media with higher glucose content are due to liberation of acids when glucose is heated in alkaline medium (Plimmer, 1938; p.263.)

Table XI gives the effect of varied concentrations of glucose added to the same basal medium as in Table X. (Medium S - without starch but with 0.1% NaCl).

Table XI.

Effect of Concentration of Glucose on Enzyme Secretion.

Medium with	Init. pH	Final pH	R.T. in Mins.	Dry wt. of Myce.
5.0% Glucose	7.3	8.3	65	3.0
4.0% "	7.4	"	35	2.4
3.0% "	7.5	"	22	2.0
2.0% "	7.8	8.1	100	1.3
1.0% "	8.0	8.0	210	0.8
0.5% "	8.1	7.9	5⅔ Hrs.	0.7

The most active enzyme secretion was found to be on a medium with 3% glucose. Variations, upwards or downwards, in glucose concentration markedly reduced activity. The secretion of enzyme was independent of the amount of mycelial growth, which increased steadily with the glucose concentration.

In Table XII, four hexose sugars were compared as sources of carbon for enzyme production. The basal medium (without starch but with 0.1% NaCl) of Tables X and XI was used.

Table XII

Effect of Four Different Hexoses on Enzyme Production

Medium with	Init. pH	Final pH	R.T. in Mins.	Dry wt. of Myce.
3% Glucose	7.5	8.2	22	2.0
3% Fructose	7.3	8.1	20	2.7
3% Mannose	7.5	7.9	25	2.4
3% Galactose	"	7.6	>24 hrs.	0.3

Galactose was quite unsuitable for either enzyme secretion or growth, whereas the other three sugars were about equally good. With all four sugars the colour of the medium changed to brown during heat sterilization.

The behaviour of glucose and sucrose, separately and together, is set out in Table XIII.

Table XIII

Effect of Sucrose on Enzyme Production

Medium with	Init. pH	Final pH	R.T. in Mins.	Dry wt. of Myce.
3.0% Glucose	7.4	8.1	25	2.1
3.0% Sucrose	8.0	8.2	80	2.9
2.5% Sucrose + 0.5% Glucose	7. 9	"	27	2.3

Sucrose, used as sole carbohydrate source, behaved like starch in giving weak enzyme solutions, and this in spite

of more vigorous growth. It is, however, a suitable form of carbohydrate if a proportion of glucose is also present. No yellow colour is formed when the medium containing sucrose only is autoclaved.

The enzymic preparations recorded in Table XIII were also tested by the viscometric method, already described in connection with Fig 1. The results (Fig. 2.) show that the enzyme produced on S medium containing sucrose and no glucose gave a viscosity of 26.0 seconds after 30 minutes, while the same viscosity was reached in 6.6 and 9.3 minutes by the enzymes produced on media containing 2.5% sucrose + 0.5% glucose, and 3% glucose respectively, this confirming the results of Table XIII. that sucrose in absence of glucose is not a suitable carbohydrate for enzyme production.

In a series of experiments glucose was replaced, partially or wholly, by citrus pectin, with the results shown in Table XIV.

Table XIV.

Effect of Pectin as Carbon Source on Enzyme Production

Medium* with	Init. pH	Final pH	R.T. in Mins.	Dry. wt. of Myce.
3.0% Glucose.	7.5	8.1	22	2.4
2.5% Glucose + 0.5% Pectin	6.8	7.7	30	"
2.0% " + 1.0% "	6.5	"	35	1.8
1.0% " + 2.0% "	5.8	"	70	1.2
2.5% pectin.	5.5	7.5	120	1.0

* S medium without starch but with 0.1% NaCl.

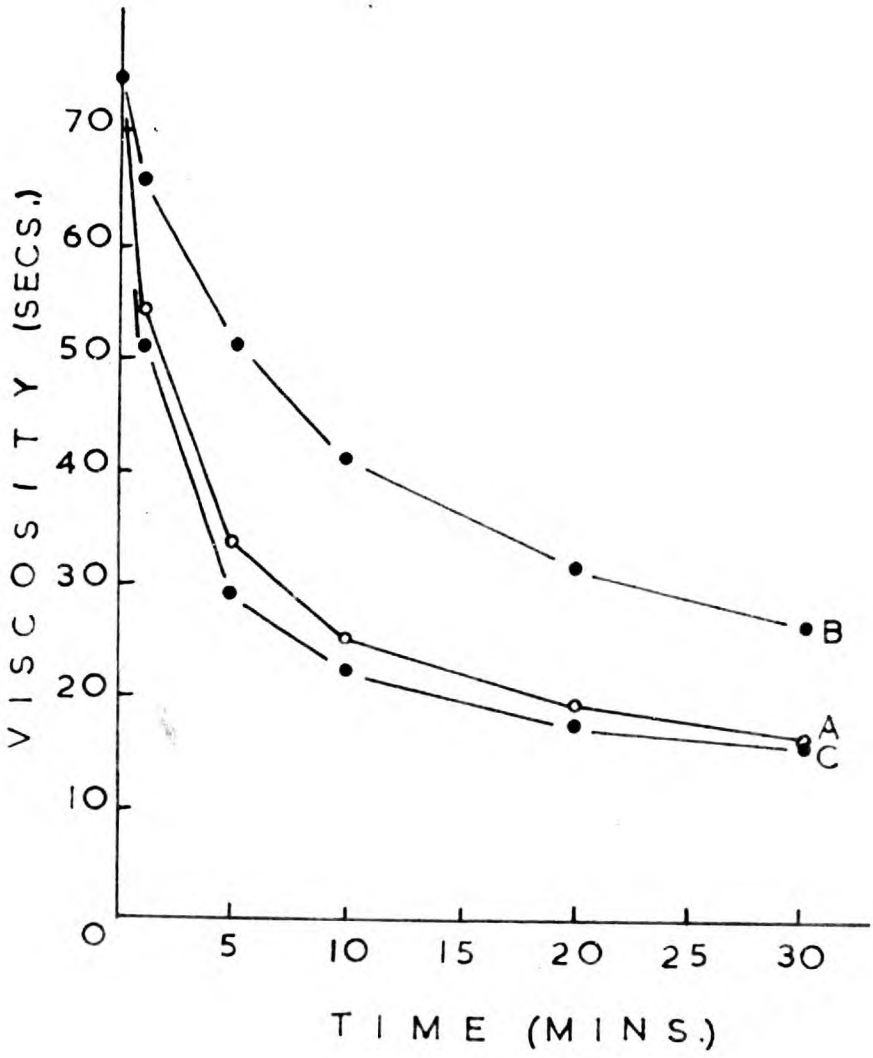


Fig. 2. Pectolytic enzyme production by P.deBaryanum in culture media containing glucose (A), sucrose (B), or glucose and sucrose both (C).

The results of Table XIV should be considered side by side with those of Table III (p. 16). The experiments of Table XIV indicate that replacement of glucose by citrus pectin gave less mycelial growth andless enzymic activity, the effects being greater the greater the degree of replacement. Pectin is thus inferior to glucose for the purpose in view. In Table III the addition of pectin to a medium containing 3% carbohydrate gave a pronounced enzyme production. It will be noted, however, that in Table XIV the basal medium contained 0.1% NaCl, whereas the latter was absent in the experiments of Table III. It is suggested therefore that the stimulating effect shown by addition of pectin in Table III was largely due to small quantities of salts, in particular chlorides, present in the pectin solution. The two experiments (Tables III and XIV) are not strictly comparable, and the results are therefore not necessarily in conflict, but further work would be required to determine the precise effect of pectin preparations on the formation of pectinase enzyme.

(e) Effect of Source and Concentration of Nitrogen.

Ashour's medium (S) contained both asparagine and peptone (each 0.45%) as sources of nitrogen. Two sets of experiments were set up to see if either of these was replaceable by any other nitrogenous compound. The replacements were made on the basis of the same total nitrogen.

Table XV shows the effect of replacing the asparagine of

Ashour's medium by equivalent amounts of six other compounds of nitrogen. The first medium in the Table ("Asparagine") is the control, viz. Ashour's medium. To all the media 0.1% NaCl was added. A similar explanation applies to the media of Table XVI where the peptone component of Ashour's medium was replaced.

Table XV.

Replacement of Asparagine by Equal Amount of Six Nitrogenous Compounds.

Medium with	Init. pH	Final pH	R.T. in Mins.	Dry wt. of Myce.
Asparagine (S)	7.8	8.0	30	1.9
Peptone*	7.5	"	70	2.5
Ammonium tartrate	7.4	5.0	80	1.5
" chloride	7.4	5.0	110	1.5
" nitrate	7.5	7.4	50	1.7
Potassium "	7.7	8.2	75	1.4
Sodium "	7.7	8.2	80	1.5

* Peptone containing 15% N.

Table XVI.

Replacement of Peptone by Equal Amount of Six Nitrogenous Compounds.

Medium with	Init. pH	Final pH	R.T. in Mins.	Dry. wt. of Myce.
Peptone	7.8	8.0	27	1.7
Asparagine	"	"	190	1.4
Ammonium tartrate	7.5	7.5	60	1.8
" chloride	7.4	7.2	90	1.5
" nitrate	7.5	7.6	110	1.6
Potassium "	7.8	8.0	80	"
Sodium "	"	"	"	"

From the above two experiments it is quite evident that both asparagine and peptone are required for most active enzyme production.

Tables XVII and XVIII, representing experiments conducted on the same general plan as the two preceding ones, show the effect of varying the concentration of asparagine and peptone (respectively) in Ashour's medium (S). All the media contain 0.1% NaCl.

Table XVII

Effect of Concentration of Asparagine

Medium with	Init. pH	Final pH	R.T. in Mins.	Dry Wt. of Myce.
No Asparagine	8.0	8.0	>180	1.8
0.12% "	"	8.1	130	2.2
0.225% "	"	"	35	2.5
0.45% " (Medium S)	"	"	30	2.1
0.6% "	"	"	150	1.9

Table XVIII

Effect of Concentration of Peptone.

Medium with	Init. pH	Final pH	R.T. in Mins.	Dry Wt. of Myce.
No Peptone	8.0	8.0	20 hrs.	1.4
0.12% "	"	8.1	200	1.6
0.225% "	"	"	120	1.8
0.45% " (Medium S)	"	"	30	2.1
0.6% "	"	"	120	1.9

It is concluded from Tables XVII and XVIII that most suitable concentration of asparagine and peptone is as set out in Ashour's medium i.e. each at 0.45%.

(f) Effect of Concentration of Potassium phosphate (tribasic) and Magnesium sulphate.

The effect of four levels of potassium phosphate (tribasic) with 0.19% magnesium sulphate and of four levels of the latter with 0.34% potassium phosphate (tribasic) isset out in Table XIX.

Table XIX.

Effect of Concentration of K_3PO_4 and $MgSO_4$.

Medium* with		Init. pH	Final pH	R.T. in Mins.	Dry Wt. of Myce.
0.19% $MgSO_4$ + 0.425% K_3PO_4		8.2	8.2	27	2.2
" + 0.34% " (control)		8.0	8.1	20	2.1
" + 0.255% K_3PO_4		7.7	"	33	2.0
" + 0.17% "		7.5	"	27	2.2
0.237% $MgSO_4$ + 0.34% "		8.0	"	20	2.0
0.19% " + " (control)		"	"	"	2.1
0.143% " + "		"	"	22	"
0.093% " + "		"	"	25	"

* All media contain 0.1% NaCl.

The controls as before, gave the most active preparations, but there was little difference between the various media as regards any of the features recorded in Table XIX.

(g) Effect of Cultural Methods on Enzyme Production.

In Table XX the effect of the volume of culture solution in each flat bottle, is set out. Ashour's medium (S) with 0.1% NaCl was used. Other cultural conditions were the same as in previous experiments.

Table XX.

Effect of Volume of Medium on Enzyme Production.

Vol. of Medium in Each Bottle.	Init. pH	Final pH	R.T. in Mins.	Dry Wt. of Myce.
10 mls	8.0	8.1	22	1.5
15 mls	"	8.1	25	1.9
20 mls	"	8.0	30	2.7
25 mls	"	8.0	40	3.5
30 mls	"	8.0	45	4.1

It was concluded from the above experiment that though mycelial growth increased with increase of volume of medium, the enzyme production was adversely affected.

In Table XXI the effect of incubation time, all other factors being the same, is set out.

Table XXI.

Effect of Incubation Period on Enzyme Production.

Incubation in Days.	Init. pH	Final pH	R.T. in Mins.	Dry Wt. of Myce.
1	8.0	7.8	>3 hrs.	0.15
2	"	7.5	90	1.5
3	"	7.9	25	2.0
4	"	8.1	23	"
5	"	"	23	"
6	"	"	33	1.9
7	"	"	35	1.7
8	"	8.0	40	1.4
9	"	"	50	1.3
10	"	"	55	1.25

From Table XXI it was concluded that maximum activity of exoenzyme and mycelial growth were reached in 3-5 days, after which the dry weight of mycelium decreased due to autolysis accompanied by fall in the activity of the enzyme. The pH at first fell slightly but reached the initial level after four days' growth.

Table XXII shows the results when cultures were grown at various temperatures ranging from 5° - 35°C. All other factors were as before.

Table XXII.

Effect of Temperature on Enzyme Production.

Temperature during Incubation	Init. pH	Final pH	R.T. in Mins.	Dry Wt. of Myce.
5°C	8.0	No Growth		
10°C	"	No Growth		
15°C	"	7.4	130	1.05
20°C	"	8.0	30	3.0
25°C	"	8.1	25	2.6
30°C	"	"	40	2.5
35°C	"	7.7	140	2.0

It is concluded from Table XXII that the most suitable temperature for enzyme production was in the neighbourhood of 25°C.

(h) General Summary of Sections (a) - (g).

The results given in Tables VI - IX and X - XIV led to the inclusion of 0.1% NaCl and the replacement of 2.5% starch and 0.5% glucose by 3.0% glucose in Ashour's medium (S). The experiments of Tables XV - XVIII confirm that the peptone and asparagine present in the S medium, and the proportions at which they were included, were about optimal for enzyme production by P.deBaryanum. From Table XIX the exact proportion of Potassium phosphate and magnesium sulphate was found to be of comparatively little significance. Further details were fixed by the experiments of Tables XX - XXII, viz. the volume of medium standardized at 15 mls per bottle, and incubation period 5 days at 25°C. The medium M, the formula of which is given on page 9, is the outcome of this work, and it was used as the basal medium when quantities of active enzyme were required.

4. Effect of Heat Sterilization of Synthetic Medium in Relation to Enzyme Production.

In all the above experiments the medium was sterilized by Damle's method, i.e. by autoclaving magnesium sulphate separately from the rest of the medium and mixing them after cooling. This type of autoclaving always resulted in the formation of a yellow colour in the medium. The present section records the results of experiments investigating the effect of sterilizing the medium in various ways on enzyme production. Attempts were

also made to identify the compounds responsible for increased enzyme production in certain heat sterilized media.

In the experiment of Table XXIII medium S was used (i.e. 2.5% starch, 0.5% glucose, 0.45% asparagine, 0.45% peptone, 0.34% K_3PO_4, and 0.19% $MgSO_4$). The media were prepared as follows:

A. All constituents autoclaved together.

B. $MgSO_4$ autoclaved separately from rest of medium.

C. K_3PO_4 " " " " " "

All culture bottles were incubated at 25°C for five days. The results obtained are shown in Table XXIII.

Table XXIII.

Enzymic Activity on S Medium Autoclaved in Three Ways.

Medium	Init. pH	Final pH	Colour after Autoclaving	R.T. in Mins.
A	7.8	8.0	Light Yellow with Precipitate	115
B	8.0	8.2	Yellow	70
C	8.1	8.1	No change.	175

Methods B and C avoided the formation of strong precipitate by magnesium sulphate. When the phosphate was separated from the other constituents no yellow colour appeared and enzyme activity was weakest. The fact that enzyme activity on A was weaker than on B may have been due to some effect of the precipitate formed in A.

Table XXIV records a similar experiment except that

0.1% NaCl was added to all media; the media A', B', C'. Correspond to A, B, C respectively of Table XXIII.

Table XXIV.

Enzyme Activity on S Medium (with 0.1%) Autoclaved in Three Ways.

Medium	Init. pH	Final pH	Colour after Autoclaving	R.T. in Mins.	Dry. Wt. of Myce.
A'	7.0	8.1	Light yellow with ppt.	60	1.9
B'	8.1	"	Yellow	30	"
C'	8.2	8.2	No change	100	2.2

The results of the above Table are similar to those of the preceding one; in general enzyme activity, however, being greater.

The results of five similar experiments, conducted at various times, are summarised in Table XXV, the notation being as in the two preceding Tables.

Table XXV.

Effect of Autoclaving (Five Experiments).

Medium	R.T. (Mins.) of Experiments. 1	2	3	4	5
A	90	80	100	100	90
B	50	45	65	60	35
C	120	100	110	110	110
A'	30	25	45	40	30
B'	25	20	30	25	25
C'	35	50	70	60	60

All these results are in accord with those of XXIII and XXIV.

Similar experiments were carried out with medium M, a modified form of S (viz. 0.5% glucose and 2.5% starch replaced by 3% glucose, together with 0.1% NaCl).

This medium was autoclaved by three methods.

a. $MgSO_4$ + remainder of medium.

b. $MgSO_4$ + K_3PO_4 + remainder of medium.

c. $MgSO_4$ + glucose + remainder of medium.

Note: The '+' sign indicates that the constituents on either side of it were autoclaved separately (so that, for example, there were three separate autoclaving in method b and c). This notation will be observed throughout this section.

The results are shown in Table XXVI.

Table XXVI.

Enzyme Production on Medium M Autoclaved in Three Ways.

Medium	Init. pH	Final pH	Colour after Autoclaving	R.T. in Mins.	Dry Wt. of Myce.
a	7.5	8.2	Light Brown	27	2.1
b	8.0	8.3	No change	75	2.35
c	8.1	8.1	"	60	2.15

Table XXVI gives the same type of results as Tables XXIII-XXV, with the further point that glucose and phosphate have to be autoclaved together for most active enzyme production. This effect is associated with the development of a yellow to brown colour. A further experiment was carried out to determine how far both were related to the degree of heating during sterilization.

In the experiment of Table XXVII medium M was used throughout, and in all the preparations the magnesium sulphate was autoclaved separately. The remainder of the medium was sterilized in variety of ways; viz. by filtering through

a Seitz. filter, by steaming for 30 minutes on three successive days, by autoclaving at 5 lbs. pressure for 20 minutes on two successive days, or by autoclaving once at 10, 15, or 20 lbs. pressure for 20 minutes.

Table XXVII

Effect of Different Methods of Sterilization.

Method of Sterilization	Colour after Sterilization	Init. pH	Final pH	R.T. in Mins.	Dry Wt. of Myce.
Filtration	No change	8.0	8.0	75	1.8
Steaming	Light Yellow	7.8	8.4	25	2.0
Autoclaving at 5 lbs	Yellow	"	8.3	"	2.2
" 10 lbs	Dark Yellow	"	"	"	2.5
" 15 lbs	Light Brown	7.5	8.2	"	1.9
" 20 lbs	Brown	7.4	7.5	35	1.6

The low activity of the first treatment shows that heating is required, but a wide range of heat treatments gives the same result. In the last treatment there is an indication of over heating, shown by reduced growth and enzyme activity with a more acidic medium.

Though it has been shown (Table XXII-XXVII) that a yellow-brown colour in the medium is associated with high production of enzyme, the following shows that this is only incidental.

Cultures on medium M were set up by three methods;

A. $MgSO_4$, K_3PO_4, and remainder of medium autoclaved separately. Medium colourless.

B. $MgSO_4$ + remainder of medium autoclaved separately. Medium light brown.

C. Medium prepared according to B was decolourized by adding 2 gms of active charcoal to 100 mls of medium and shaking for an hour. This was then sterilized by filtration which also removed the charcoal.

The results are shown in Table XXVIII.

Table XXVIII.

Effect of Decolourization on Enzyme Production.

Medium	Init.pH	Final pH	R.T. in Mins.	Dry wt. of Myce.
A	8.0	7.9	90	1.8
B	7.4	8.2	25	2.1
C	7.4	8.2	30	1.6

The somewhat reduced growth and enzymic activity on (c) as compared to (b) may have been due to adsorption of some nutrient components by the charcoal.

The possibility that the effects of autoclaving glucose with phosphate were due, not to the phosphate as such, but simply to alkali was investigated with the results shown in Table XXIX. Medium M was used in all cases, and in the preparation the magnesium sulphate was autoclaved separately.

Table XXIX.

Effect of pH on Enzyme Production.

Medium autoclaved with	Init. pH	Final pH	Colour after autoclaving	R.T. in Mins.	Dry Wt. of Myce.
K_3PO_4	7.5	8.2	Light Brown	22	2.1
K_2HPO_4	7.2	7.8	Yellow	25	"
KH_2PO_4	5.2	7.1	No change	65	2.3
K_3PO_4 + 0.1% $CaCO_3$	7.4	8.2	Deep Yellow	25	2.2
K_2HPO_4+ "	7.2	8.0	Light Yellow	25	2.1
KH_2PO_4+ "	7.0	7.9	Very Light Yellow.	50	2.2

The medium with potassium dihydrogen phosphate had an initial pH of 5.2 and produced a colourless medium of low capacity for producing enzyme; when autoclaved with $CaCO_3$ it gave a slightly coloured medium and intermediate enzyme activity.

The stimulating effect under consideration was further analysed in the experiment recorded in Table XXX. Medium M was used, its constituents being autoclaved separately according to the scheme below:

A. $MgSO_4$ + Rest.

B. $MgSO_4$ + Glucose + Rest

C. " + " + " + 1 ml. of 7.5% glucose autoclaved with 0.1% K_3PO_4

D. " + " + " + " with 0.1% Na_2CO_3

E. " + " + " + " with 0.1% NH_4CO_3

F. " + " + " + " with 0.1% NaOH

Table XXX.

Medium	Init.pH	Final pH	R.T. in Mins.	Dry Wt. of Myce.
A	7.5	8.1	25	2.6
B	8.1	7.9	75	2.1
C	8.2	8.3	25	2.7
D	"	8.2	50	2.6
E	8.1	7.7	65	2.8
F	"	8.3	40	2.3

Table XXX shows that the best results were obtained with A and C, i.e. by autoclaving glucose with phosphate; but a distinct stimulation was observed with D and F, so that some of the effect was obtained merely by heating glucose with alkali. It is not excluded that by appropriate heating glucose with alkali of the correct strength, the full effect might be obtained.

These results already suggested that the stimulating effect was not due to the action of phosphoric esters of

glucose as was postulated by Damle (l.c.). The question was taken up further by a chromatographic examination of a solution containing 7.5% glucose and 0.34% K_3PO_4 after autoclaving at 15 lbs. pressure for 20 minutes. The method used for the identification of phosphoric esters of sugars is that of Bandurski and Axelrod (1951) and is briefly as follows.

Whatman No. 1 filter paper was cut in 20 x 40 cms. pieces. A line at a distance of 5 cms. from one edge was drawn across the width. 0.01 mls. of an autoclaved glucose/K_3PO_4 solution and 0.002 ml. each of 1% K_3PO_4, 1% glucose-1-phosphate, and 1% 3-phosphoglycerate were spotted on the line at intervals of 4 cms. After drying the paper was rolled into a cylinder and an iron wire (about 0.25 cms. in diameter) was punched across one end away from the spots. A glass cylinder, 10 cms. diameter and 50 cms. high, was taken and 30 mls. of developer (80 mls. methanol, 15 mls 88% formic acid, and 5 mls. water) placed in it. A glass plate was used to cover the opening of the cylinder and from its lower surface the paper cylinder was suspended by placing a strong magnet on the upper surface. The paper cylinder was not allowed to touch the wall of glass cylinder. After equilibration for 2 hours at 20°C, the magnet was removed and the paper cylinder allowed to dip into the developing liquid. After about six hours at 2°C, the developer had reached the upper end of filter paper by capillary action. The paper was then removed from the glass cylinder and dried at room temp-

erature. It was sprayed with the Hanes-Isherwood reagent (5 mls. perchloric acid, 25 mls. 4% ammonium molybdate, 10 mls. N Hcl, and 60 mls. water), and after drying heated to 85°C. for 1-2 minutes. The paper was then exposed to ultra violet radiation at a distance of 10 cms. for 10 minutes. After this treatment, organic phosphates give bluish spots while inorganic phosphate spots turned yellowish in colour.

The results, which are illustrated in Fig. 3, show that no organic phosphates are formed by heating glucose in presence of K_3PO_4.

It was realised that the change of colour in the medium was due to caramelization of glucose which takes place on heating it with alkal (K_3PO_4). Five grms. of glucose were therefore dry-heated in a crucible for 20 minutes in a hot air oven at 150-155°C. The glucose melted at this temperature, collected at the bottom of crucible, turned brown and became solid after cooling. This 'caramel' was dissolved in distilled water to make 10 mls. of 50% caramel solution. This solution was autoclaved separately and added to the medium when required.

The following media were made up with medium M, each series of components (separated by '+' sign) being sterilized separately.

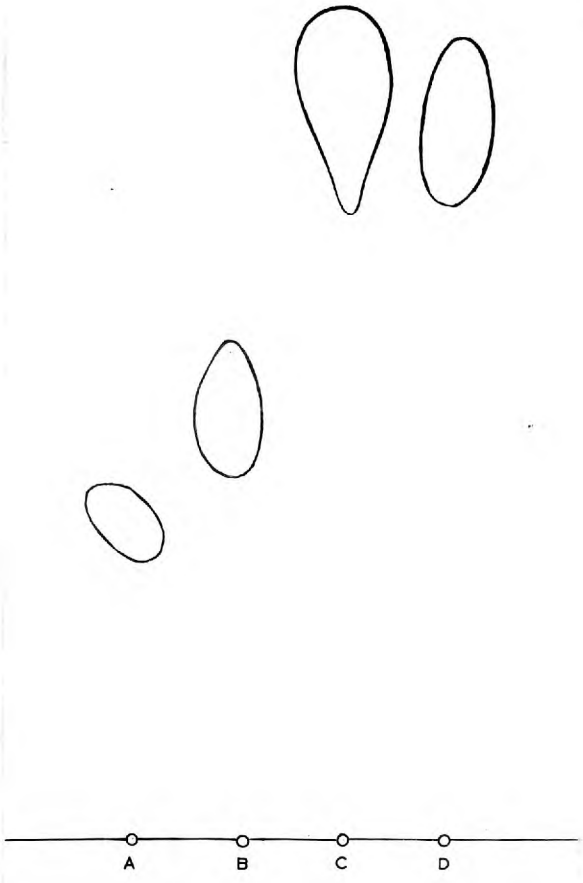


Fig. 3. Chromatographic separation of phosphates.

A. glucose-1-phosphate; B. 3-phosphoglycerate;

C. autoclaved glucose/K_3PO_4 solution;

D. K_3PO_4.

A. $MgSO_4$ + Rest

B. " + K_3PO_4 + Rest

C. " + " + " + 0.25 mls. of 7.5% glucose autoclaved with 0.1% K_3PO_4

D. " + " + " + " decolouried with active charcoal

E. " + " + " + 0.25 mls. of 50% caramel

F. " + " + " + " decolourised.

The results obtained by the above experiment are shown in Table XXXI below.

Table XXXI.

Effect of Caramel on Enzyme Production.

Medium	Init. pH	Final pH	R.T. in Mins.	Dry Wt. of Myce.
A	7.5	8.0	30	2.0
B	8.1	8.1	65	2.1
C	7.9	8.0	35	3.1
D	8.0	8.1	35	3.0
E	7.9	8.0	25	2.5
F	8.0	"	25	2.2

It was thus strongly suggested that enzyme production is stimulated by a breakdown product of glucose which is formed in a variety of ways - (a) by autoclaving in presence of alkali and in particular of K_3PO_4, (b) by heating dry glucose at 150-155°C. During this breakdown, caramelization, a yellow colour is formed but its presence is of no consequence

to the stimulating effect of this solution on enzyme production.

Table XXXII shows the effect of different additions of caramel solution to medium M.

Table XXXII.

Effect of Different Concentrations of Caramel on Enzyme Production.

Medium	Init. pH	Final pH	R.T. in Mins.
$MgSO_4$ + M	7.5	8.2	25
" + K_3PO_4 + M	8.0	"	75
" + " + " + 0.01% Caramel	"	"	60
" + " + " + 0.05% "	"	"	53
" + " + " + 0.1% "	"	"	40
" + " + " + 0.25% "	"	"	35
" + " + " + 0.5% "	"	"	25
" + " + " + 1.0% "	"	"	25

It was concluded from the above Table that the optimum concentration of caramel lies somewhere between 0.25 - 0.5%.

Various experiments were set up from time to time to test the effect of caramel on enzyme secretion, the former being added in 0.33% concentration to medium M. The results of eight such experiments are summarised in Table XXXIII.

Table XXXIII.

Effect of 0.33% Caramel on Enzyme Production.

Medium	R.T. (in Mins) of Experiments							
	1	2	3	4	5	6	7	8
$MgSO_4$ + M	25	25	25	30	35	35	30	27
" + K_3PO_4 + M	80	100	75	65	80	60	65	65
" + " + " + Caramel	25	45	25	25	30	22	27	25

The activity of the enzyme secreted on the media of Table XXXIII was also tested by viscometry. The results illustrated in Fig. 4, show that the enzyme production on a medium where K_3PO_4 was separately autoclaved, was much weaker compared with media where K_3PO_4 was autoclaved with other components, or where 0.33% caramel was added.

An attempt was made with the help of descending paper chromatography to identify the changes taking place when glucose is in the dry condition or in alkaline solution. 0.004 mls. of each solution was applied to a sheet of Whatman No. 1 filter paper. This was allowed to eequilibrate in a chamber containing the lower layer of a 4:1:5 mixture of n-butanol-acetic acid-water. The upper layer of this mixture was then added to the trough and after developing for 20 hours at room temperature (15°- 20°C), the sheet

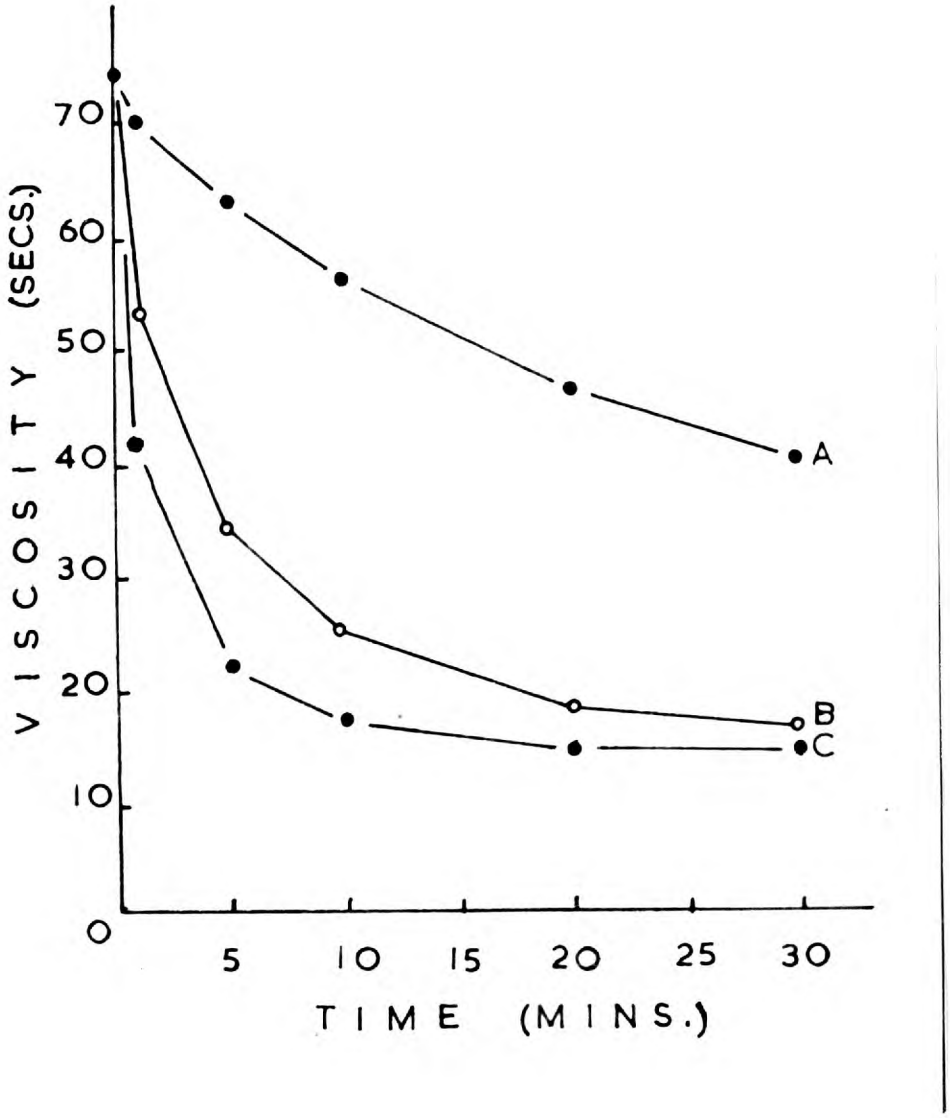


Fig. 4. Effect of caramel and method of autoclaving on pectolytic enzyme production by P.deBaryanum.

A - $MgSO_4$ + K_3PO_4 + M; B - $MgSO_4$ + M;

C - $MgSO_4$ + K_3PO_4 + M + 0.33% Caramel.

was air dried, sprayed with a benzidine reagent (0.5 grm. benzidine, 10 mls. acetic acid, 10 mls. 45% trichloroacetic acid, and 100 mls. 95% ethanol) and heated at 95°-100°C. for 3-5 minutes. This reagent gave brown, yellow and pink colour reactions with various sugars or their derivatives.

The sugar solutions tested and the results obtained are illustrated in Fig. 5.

The following conclusions may be drawn from Fig. 5:

1. Some fructose, mannose and possibly glyceraldehyde and dihydroxyacetone are formed by autoclaving glucose solution in the presence of K_3PO_4 or NaOH.

2. Some fructose, possibly glyceraldehyde and dihydroxyacetone, and higher molecular weight derivatives of glucose are formed when glucose is dry heated at 150-155°C. for 20 minutes.

On the basis of these observations, an experiment was set up to study the effect of fructose and mannose on enzyme secretion. The media given below were prepared, and the results are shown in Table XXXIV.

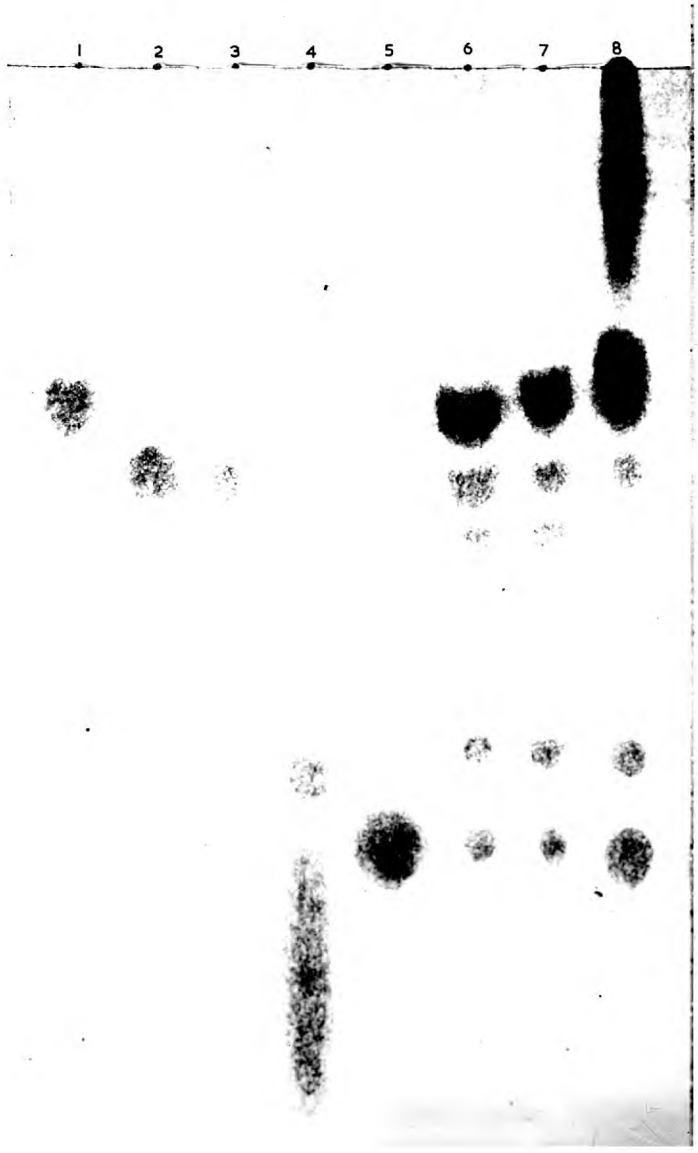


Fig. 5. Chromatographic separation of sugars.
spots 1 - glucose, 2 - mannose, 3 - fructose,
4 - glyceraldehyde, 5 - dihydroxyacetone,
6 - autoclaved glucose/K_3PO_4 solution, 7 - autoclaved
glucose/NaOH solution, and 8 - caramel.

a. $MgSO_4$ + Rest of M.

b. " + 3% Glucose + Rest of M.

c. " + 2% " + " + 1% Mannose.

d. " + " + " + 1% Fructose

e. " + 1% Glucose + " + " + 1% Mannose.

Table XXXIV.

Effect of Mannose and Fructose on Enzyme Production.

Medium	Init. ph	Final pH	R.T. in Mins.	Dry Wt. of Myce.
a	7.5	8.2	25	2.1
b	8.0	"	70	2.0
c	"	"	60	1.8
d	"	"	60	1.9
e	7.9	"	50	2.2

From the above results it was concluded that though a mixture of glucose and mannose, glucose and frustose, or glucose, fructose and mannose were slightly better than glucose alone, they were inferior to glucose autoclaved with alkaline phosphate.

Glyceraldehyde and dihydroxyacetone (supplied by L. Light & Co.) were similarly tested in a series of media as follows:

A. $MgSO_4$ + Rest of M.

B. " + K_2PO_4 + Rest of M.

C. " + " + " + 0.1% Fructose.

D. " + " + " + 0.1% Glyceraldehyde.

E. " + " + " + 0.1% Dihydroxyacetone.

F. " + " + " + " + 0.1% Glyceraldehyde

G. " + " + " + " + " + 0.1% Fructose

H. " + " + " + 0.33% Caramel.

The results are shown in Table XXXV.

Table XXXV.

Effect of Trioses on Enzyme Production.

Medium	Init. pH	Final pH	R.T. in Mins.
A	7.5	8.1	30
B	8.0	8.2	65
C	"	"	50
D	"	"	30
E	"	"	45
F	"	"	33
G	"	"	37
H	"	"	25

The Enzyme activity of media A-H of Table XXXV was also tested by viscometry and the resultsare illustrated in Fig. 6.

Table XXXV and Fig. 6 show that the addition of

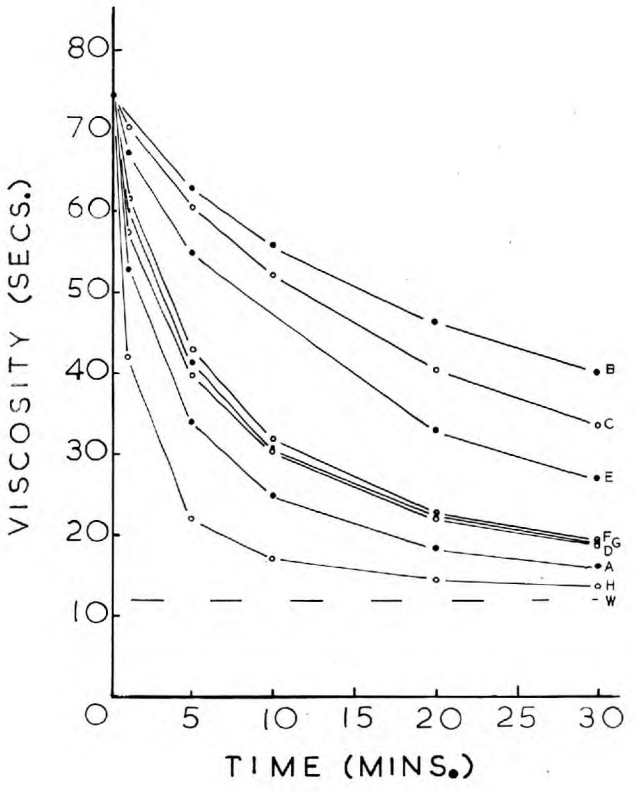


Fig. 6. Effect of addition of various sugars to medium M on pectolytic enzyme production by P.deBaryanum. W - water; A to H - as on page 55.

glyceraldehyde to the medium considerably stimulated enzyme secretion. In the chromatogram of caramel (Fig. 5) there were indications of fructose and dihydroxyacetone in addition to glyceraldehyde. But when a mixture of glyceraldehyde, dihydroxyacetone, and fructose was used, it proved to be inferior to glyceraldehyde alone. However, the possibility of more active enzyme production by using appropriate concentration of these three sugars still remains.

To determine the effect of glyceraldehyde and other components of caramel on enzyme secretion, the following experiment was carried out:

On each of 6 spots on a filter paper sheet were put 0.02 mls. of 50% caramel solution. To avoid formation of large spots, only 0.005 mls. of the solution was added at one time. The chromatogram was then developed for 24 hours in the way already described on page 50 . The sheet was then dried at room temperature for 24 hours and then at 35°C. for another 24 hours to remove traces of the developing mixture. It was then cut longitudinally to remove one of the six spots, and the latter sprayed with the benzidine reagent to mark the position of these components on the unsprayed sheet. Each division so formed was cut into small strips and sterilized separately in dry Petri dishes by autoclaving at 15 lbs. pressure for 20 minutes. These strips were then transferred into flats containing the medium. It was supposed that this operation gave the same concentration of each component in which it was

present in 0.1 ml. of 50% caramel solution. The unsprayed sheet was divided into six parts containing the following sugars:

A. Five cms. strip from front end of the sheet containing no sugars (control).

B. Portion containing high molecular weight derivatives of glucose.

C. " " glucose.

D. " " fructose and sheet up to the base of trioses.

E. " " trioses (glyceraldehyde & dihydroxy-acetone).

F. Five cms. broad strip after E portion.

The activity of the enzyme was tested by maceration and viscometry and the results are shown in Table XXXVI.

Table XXXVI.

Effect of Different Components of Caramel

Medium	Init. pH	Final pH	R.T. in Mins.	* Decrease in Viscosity. after 30 Mins.
$MgSO_4$ + Rest	7.6	8.2	30	88.6
" + K_3PO_4 + Rest	8.0	"	85	48.5
" + " + " + A	"	"	65	55.2
" + " + " + B	"	"	47	86.3
" + " + " + C	"	"	85	63.7
" + " + " + D	"	"	75	47.0
" + " + " + E	"	"	40	72.8
" + " + " + F	"	"	85	48.5
" + " + " + 0.33% caramel	"	"	27	89.3

* As % of total possible loss in viscosity.

It is concluded from the above Table that portion E of Chromatogram (containing trioses) caused greatest stimulation of enzyme secretion on the basis of maceration (in comparison to other parts of the chromatogram). But portion B is most effective when loss of viscosity is used as a measure of activity. The reasons for this are not known and require further examination.

Together with the previous data it is concluded that during caramelization of glucose (either by dry heating, or by heating in alkaline solution), some fructose, glyceraldehyde and dihydroxyacetone (with alkali mannose as well) are formed. These when added to the medium in a certain proportion, markedly stimulate enzyme production. On the evidence available, glyceraldehyde seem to be the substance mainly responsible for this stimulating effect.

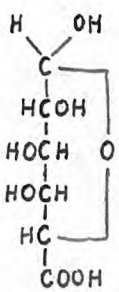
Second Part. STUDIES ON PECTOLYTIC ENZYMES PRODUCED BY PYTHIUM DEBARYANUM IN SYNTHETIC CULTURE MEDIA.

A. TERMINOLOGY OF PECTIC SUBSTANCES AND PETOLYTIC ENZYMES.

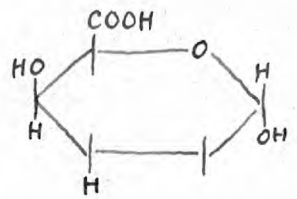
It is now generally accepted that plant tissues contain a large proportion of pectic substances and that some parasites attack by secreting pectolytic enzymes. In renent years considerable advances have been made in the study of pectic substances and of the enzymes which are responsible for their breakdown. The modern views on the topic have been comprehensively reviewed by Kertesz (1951), but it is essential for the proper understanding of the work to be described that they should be briefly stated here.

"Pectic substances is a group designation for those complex, colloidal carbohydrate derivatives which occur in, or are prepared from plants and contain a large proportion of anhydrogalacturonic acid units which are thought to exist in a chain-like combination. The carboxyl groups of polygalacturonic acids may be partly esterified by methyl groups and partly or completely neutralised by one or more bases.

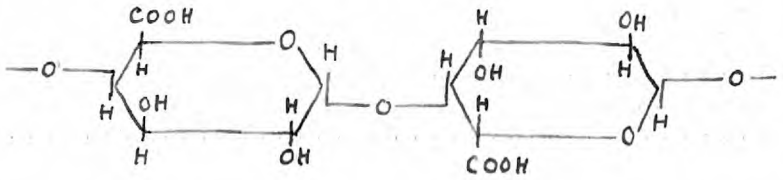
The following shows the structure of galacturonic acid and the suggested structure of polygalacturonic acid (pectic acid):-



or



D - Galacturonic acid



Chain of polygalacturonic acid (pectic acid) without end groups.

It would be fair to mention here that the simple chain-like structure of pectic substances has been questioned from time to time by many workers. The most important view is that of Kertesz (1939) who suggested that simple chain-like polygalacturonic acid molecules are aggregated into a complex structure. He postulated the structure $\left[(G)_m \right]_n$ for pectin in which $(G)_m$ is polygalacturonic acid and n units of polygalacturonic acids unite to form a secondary aggregate.

The following pectic substances have been distinguished.

(1) "Protopectin is the water-insoluble parent pectin substance which occur in plants and which, upon restricted hydrolysis, yields pectinic acids".

(2) "Pectinic acids are colloidal polygalacturonic acids containing more than a negligible proportion of methyl ester groups".

(3) "Pectins are those water-soluble pectinic acids of varying methyl ester content and degree of neutralization which are capable of forming gels with sugar and acid under suitable conditions".

(4) "Pectic acid. This term is applied to pectic substances mostly composed of colloidal polygalacturonic acids and essentially free from methyl ester groups. The

salts of pectic acid are either normal or acid pectates".

None of the above described definitions is exact as the minimum molecular size of pectic polygalacturonic acid or the minimum molecular weight at which the polygalacturonic acid becomesa pectic acid is not yet known.

Pectolytic enzymes also are not yet fully understood, because of the complex macromolecular structure of pectic substances. There may be a large number of such enzymes, but only four have been described by Kertesz (1951) and of these only two are definitely known (2 & 4 below).

(1) Protopectinase is the name given to an enzyme which hydrolyses or dissolves protopectin, with resultant separation of plant cells from each other, a process usually spoken of as maceration. The action of protopectinase has so far been demonstrated only by its action on plant tissues and an exact chemical definition of the reaction is not possible because of the unclarified chemical nature of protopectin. There is now a growing tendency towards the view that protopectinase is identical with pectin-polygalacturonase (2, below). This assumption rests on the fact that all preparations of protopectinase which have been examined have been found to contain polygalacturonase and both behave in such a similar manner that it is not possible to separate them.

(2) Pectin-polygalacturonase or polygalacturonase (PG) has previously been described as pectolase and pectinase. Polygalacturonase enzyme is characterised by its hydrolysing

action on the 1,4 glycosidic linkage in the polygalacturonic acid skeleton of pectic or pectinic acids with the resulting formation of polygalacturonic acids of smaller molecular sizes and finally of (mono-) galacturonic acid. PG has been found to have exceedingly slow, if any, hydrolysing action on fully esterified (methyl) polygalacturonic acid and hence the true substrate for PG is pectic acid and not pectinic acid.

(3) Pectic acid 'Depolymerase'. The existence of this enzyme is not yet fully established and has only been demonstrated in tomato fruit. This enzyme is almost entirely without action on pectinic acids but very active on pectic acid, which it partially hydrolyses but never to the end point of (mono-) glacturonic acid.

(4) Pectin-methyl esterase or pectinesterase (PE) is the enzyme which catalyses the hydrolysis of the methyl ester groups in pectinic acids and pectin and the end products are methyl alcohol and pectic acid. It has no effect on the molecular chain of pectin.

Apart from the above four enzymes, there is evidence of one or more pectolytic enzymes. An enzyme, called poly-methyl-galacturonase', has lately been described (Seegmiller & Jansen, 1952). This enzyme is very active on pectins in comparison to pectic acids and does not break down the pectin chain to (mono-) glacturonic acid, i.e. it distinctly differs from PG or pectic-acid depolymerase. A more or less similar

enzyme has been obtained from cultures of Neurospora crassa (Roboz, Barratt & Tatum, 1952) and called 'pectin-depolymerase' which is very much similar to poly-methyl-galacturonase, but attacks both pectin and pectic acid.

B. GENERAL MATERIAL AND METHODS

Organisms Tested

The three organisms used were *Pythium de-Baryanum*, *Botrytis cinerea* and *Bacterium aroideae*. All three were obtained from the stock cultures of Plant Pathology Laboratory, Imperial College of Science and Technology, London. Cultures of *Pythium* were maintained on potato-dextrose-agar, those of *B.cinerea* and *B.aroideae* on potato-extract-agar. The main work was done with *Pythium*, but *Botrytis* and *Bacterium* were used for comparison in some cases.

Media and Cultural Methods

The preparation of *Pythium* enzyme has already been described (p. 8 and 9). For *Botrytis* the following medium was used:-

Glucose	20.0 gms.
Asparagine	5.0 gms.
KH_2PO_4	1.0 gms.
$MgSO_4 . 7H_2O$	0.5 gms.

made up to 1 litre with distilled water.

Each bottle containing 30 mls. of medium was inoculated with a large number of spores. For this purpose a single vigorously sporing slope in a 15 x 2 cms. test tube was used for each flask. Sterile distilled water (about 5 mls.) was added to the slope, the spores rubbed off with a sterile loop,

and the suspension poured into the culture flask. The cultures were maintained for 4-5 days at 20°C after which the mycelium was removed and liquid freed from fragments of mycelium by centrifuging. This supernatant was used as B.cinerea* enzyme.

For Bacterium aroideae*, the same medium as for B.cinerea was used, except that 1% $CaCO_3$ was added. Each bottle containing 30 mls. of medium was inoculated directly from the agar slope by means of a sterile loop. The cultures were maintained for 4-5 days at 25°C and liquid was afterwards centrifuged at 8,000 r.p.m. for 5 mimutes to remove the bacterial cells. The supernatant thus obtained was used as B.aroideae enzyme.

Generally a fresh supply of enzyme was used for each experiment; sometimes it was stored at 4°C under toluene, but not longer than five days.

Pectic Substances Used.

Three commercial pectic substances were used in this work:

a. Citrus pectin - From California Fruit Growers Exchange, California, U.S.A. (Approximate methoxyl groups by weight 10.5%).

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* For most of the B.cinerea and B.aroideae enzyme solutions used in this work, the writer is indebted to Messrs. S.G. Fushtey and W.R. Jarvis.

b. Pectic acid - From California Fruit Growers Exchange.

c. Sodium polypectate (= Sodium-ammonium-pectate)- From California Fruit Growers Exchange. (Approximate ammonium 1.3%, Sodium 4.2%).

In order to ensure their purity the first two substances were washed first with 80% ethyl alcohol acidified with hydrochloric acid, and then with 90% ethyl alcohol. They were then dried under reduced pressure in a desiccator and then at 30°C for 12 hours.

Sodium pectate was prepared in the laboratory either from pectin or pectic acid. The three methods used are described in Section C 1 (c) below.

Measurement of Enzyme Activity.

Protopectinase activity was tested on plant tissues by the method described by Brown (1915), and mentioned on page 10. The activity of protopectinase enzyme is represented by the value $\frac{100}{R.T.}$ (R.T. = maceration time in minutes), or in terms of arbitary enzyme units.

Polygalacturonase was measured by three methods:

a. By reduction in viscosity of pectin or pectic acid solutions; or by change in optical rotation of pectin solutions.

b. by detection of end-products chromatographically.

c. by estimation of reducing groups formed.

Pectinesterase activity was measured by determining the carboxyl groups liberated.

The pH of solutions was tested either colorimetrically or with a pH - meter. Sorensen phosphate buffer was used when required.

C. EXPERIMENTAL RESULTS.

1. Comparison Between Pectolytic Enzymes Secreted by Pythium deBaryanum, Botrytis cinerea, and Bacterium aroideae.

(a) Protopectinase.

The protopectinase activity was estimated in preparations from cultures of P.deBaryanum, B.cinerea, and B.aroideae by measuring the maceration time (R.T.). The results are shown in Table XXXVII.

Table XXXVII.

Protopectinase Activity in Three Enzyme Preparations.

Source of enzyme	R.T. in Mins.
P.deBaryanum	25
B.cinerea	17
B.aroideae	12

It is evident from the above results that all the three fungi secrete protopectinase enzyme.

(b) Pectin-esterase (PE).

Pectin-esterase catalyses the hydrolysis of methyl-ester groups in pectin and pectinic acids, liberating free carboxyl groups. For the estimation of PE therefore, carboxyl groups were estimated by the method described by Kertesz (1951, P.362), which in short is given below:

Five mls. of a 1% pectin solution was taken in a glass stoppered Erlenmeyer flask and to it the following were added: 0.1 N NaOH to bring pH to 6.2, 0.1 Mls. of methyl red as indicator, distilled water to bring the volume to 8.0 mls. and then 2.0 mls. of the enzyme solution (pH 6.2). The mixture was kept at 25°C. Any fall of pH was indicated by a change of colour and was readjusted by adding 0.1 N NaOH.

No PE activity was noted in the enzyme solution from P.deBaryanum cultures in a period of 24 hours. Tests for PE were also made at pH 7.5-7.8 (cresol red and meta-cresol purple as indicators), but they also gave negative results.

Solutions from cultures of B.cinerea and B.aroideae were also tested. The former showed PE activity within 10 minutes, but no activity was noted in the latter even after 24 hours. No attempt was made to determine the PE activity of Botrytis preparations quantitatively.

Thus under conditions where pectinesterase enzyme could be detected after 10 minutes with solutions from

B.cinerea, no such effect was seen with P.deBaryanum or B.aroideae even after 24 hours.

(c) Polygalacturonase (PG).

The pectolytic enzyme preparations of P.deBaryanum, B.cinerea, and B.aroideae were compared for the presence of PG by several methods.

Viscosity.

The effect of the above enzyme preparations on the viscosity of solutions of the following five substrates was studied.

A. 1% pectin, source and purification cited on page 66 (A of Figs. 7-9).

B. 1% sodium polypectate, source and purification cited on page 66 (B of Figs. 7-9).

C. 1% sodium pectate prepared by neutralising pectic acid with sodium hydroxide. Two gms. of pectic acid was suspended in 100 mls. of water and dissolved by adding 0.1N NaOH solution. The pH of the solution was not allowed to rise above 8.5 at any stage of the operation. When the pH of the solution became steady, distilled water was added to bring the final volume to 200 mls. (C of Figs. 7-9).

D. 1% sodium pectate prepared by de-esterifying pectin

solution by the action of pectin-esterase* (PE). For this purpose 0.5 ml. of a PE preparation was added to 50 ml. of a 2% pectin solution. The pH was maintained between 6.5-7.0 by addition of 0.1 N NaOH until no fall in pH was further noted. Distilled water was then added to make a 1% solution (D of Figs. 7-9).

---

* Pectin-esterase was prepared in the laboratory in the following way: 160 gms. of fresh tobacco leaves, free from midrib, were homogenized in a Waring blendor using a small quantity of distilled water. It was collected on a Buchner funnel over 3 layers of Whatman No. 1 filter paper and the liquid discarded. The tissue was now suspended in 100 mls. of 0.2 M Na_2HPO_4 solution and left overnight at 4°C, after which the liquid was pressed out and to it ammonium sulphate was added to give 30% saturation at room temperature. The solution was left for 1 hour at 4°C. after which it was cleared by centrifuging, the precipitate being discarded. More ammonium sulphate was added to give 65% saturation. The precipitate, which formed within one hour was collected on a thin cake of Hyflosupercel on a Buchner funnel. The cake with precipitate was then suspended in 20 mls. of 0.2 M NaCl and was centrifuged to recover the supernatant which was then used as a source of PE.

E. 1% sodium pectate prepared by de-esterifying pectin solution with sodium hydroxide. For this purpose sufficient 0.1 N NaOH solution was added to a 2% pectin solution to bring the pH to 10-11. More alkali was added, from time to time, until the pH became steady at 10. Distilled water was then added to make a 1% solution (E of Figs. 7-9).

The enzyme preparations of P.deBaryanum, B.cinerea, and B.aroideae were made in the usual manner and, when tested by the maceration test at their respective optima, gave Reaction Times of 25, 17, and 12 mimutes respectively.

Viscosity determinations with Pythium enzyme preparations were as described on page 23. Similar methods were used with B.cinerea and B.aroideae preparations, but here 1.0 mls. of enzyme was used for each test and the activity was tested at pH 6.0 and 8.3 respectively. The results are illustrated in Figs. 7-9.

From Figs. 7, 8, and 9 it is concluded that all the three preparations reduced the viscosity of pectin at much the same rate. However, they acted differently on pectate solutions.

The P.deBaryanum preparation reduced the viscosity either very slightly or not at all (B, C, D, and E; Fig. 7). The B.cinerea preparation on the other hand, acted upon each pectate solution more rapidly than the pectin solution (Fig. 8).

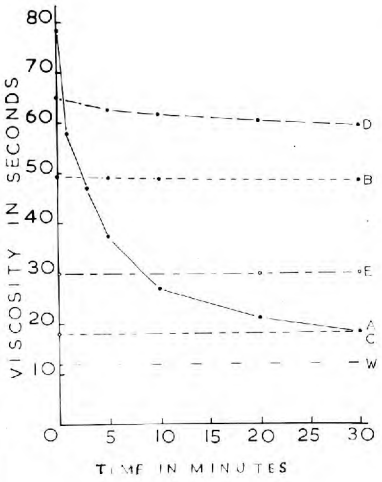

FIG. 7

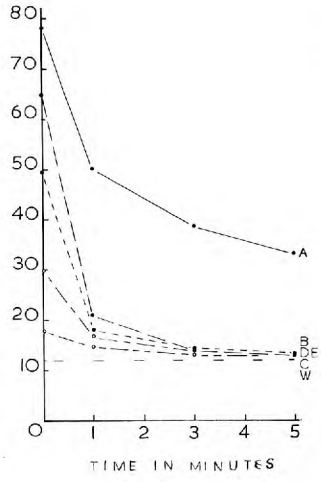

FIG. 8

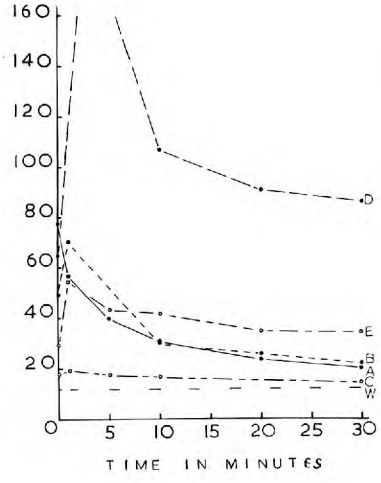

FIG. 9

Effect of pectic enzymes on viscosity ~~solution~~ of five pectic substances.

Fig. 7. Enzyme from cultures of P.deBaryanum.

Fig. 8. " " " " B.cinerea.

Fig. 9. " " " " B.aroideae.

A - pectin, B - sodium-polypectate, C - sodium-pectate prepared from pectic acid, D - sodium pectate prepared from pectin by the action of PE, E - sodium pectate prepared from pectin by the action of NaOH; W - water.

The first effect of adding the B.aroideae preparations to pectate solutions was a distinct increase in the viscosity (Fig. 9) presumably owing to the formation of calcium pectate (calcium is present in the B.aroideae preparation). Later, however, the viscosity falls but the results are easily compared with those of B.cinerea owing to the complication caused by calcium.

Optical Rotation.

The effect of enzyme solutions was also examined by measuring changes in the optical rotation of the reacting solutions. A solution of pectin with autoclaved enzyme showed a specific rotation of about +200°. The specific rotation of (mono-) galacturonic acid was about +60°. Fig. 10 shows the effect of P.deBaryanum (A), B.aroideae (B), and B.cinerea (C), enzymes on pectin solutions. As in the viscosity tests, the first two enzymes were tested at pH 8.3, and the last one at pH 6.0. The observations were made at 25°C. over a period of 24 hours. The Line D (Fig. 10) gives the theoretical end point at which all the pectin has been broken down to (mono-) galacturonic acid.

As seen from Fig. 10, only B.cinerea enzyme (c) causes a significant reduction in the specific rotation of the pectin solution.

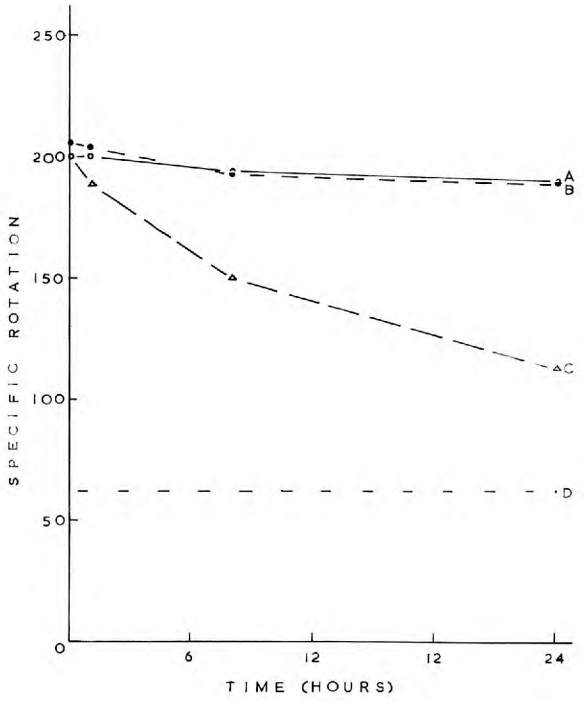

Fig. 10. Effect of pectic enzymes on specific rotation of pectin solutions.

A, B, & C - enzyme preparations from cultures of P.deBaryanum, B.aroideae, and B.cinerea respectively; D - galacturonic acid.

Chromatography.

The breakdown products of pectin by the action of P.deBaryanum, B.cinerea, and B.aroideae enzymes were examined chromatographically. Test solutions were made up as mentioned in connection with Fig. 10. The method used was similar to those described on page 50, but the time of development was increased to 48 hours. The results are illustrated in Fig. 11.

It is clear from Fig. 11 that no galacturonic acid or intermediate products are formed from treatment of pectin with P.deBaryanum or B.aroideae enzyme preparations. The B.cinerea enzyme solution, however, gave in addition to (mono-) galacturonic acid, three poly-galacturonic acid compounds of different molecular weight.

Reducing groups.

The effect of P.deBaryanum enzyme was followed by determining the increase in reducing groups in pectin solution at various stages of the reaction. The method used was as follows:-

To 5 mls. of a 1% pectin solution were added, 0.1 N NaOH to bring the pH to 8.3, 1.0 mls. of a phosphate buffer solution and distilled water to bring the volume to 8 mls, and finally 2.0 mls. of an enzyme solution. Samples of 2.0 mls. were tested from time to time by adding 0.4 mls. of 1M Na_2CO_3 solution followed by 2.0 mls. of 0.1 N iodine

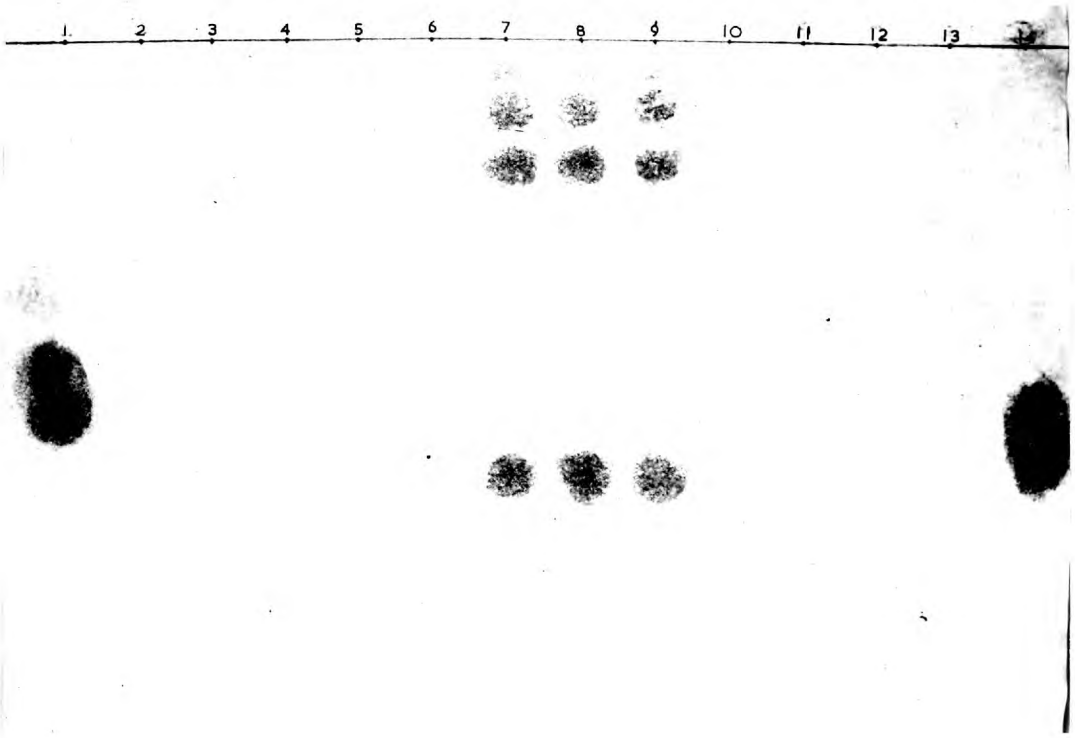

Fig. 11. Chromatographic separation of breakdown products of pectin by pectic enzymes.

spots: 1 & 14 - galacturonic acid; 2 - P.deBaryanum enzyme + pectin solution which had been allowed to react for 1 hr.; 3 - the same after 24 hrs.; 4 - the same after 48 hrs.; 5 - the same as 4 except that the amount of enzyme was doubled after 24 hrs.; 6 to 9 - similar to 2 - 5 but for B.cinerea; 10 to 13 - similarly for B.arroideae.

solution. This mixture was left for 20 minutes at room temperature and then 0.8 mls. of 2 M H_2SO_4 was added. The excess iodine was then titrated with 0.01N sodium thiosulphate solution with starch as indicator. Reducing groups were calculated as equivalent mgs. galacturonic acid and the results are shown in Table XXXVIII.

Table XXXVIII.

Formation of Reducing Groups by the Action of P.deBaryanum Enzyme.

Time in Hours	Equivalent mgs. Galacturonic acid.
0	0.0
1	0.27
4	0.66
24	0.66

A quantity of 2.0 mls. of 0.5% pectin solution corresponds approximately to 10 mgs. of galacturonic acid if completely hydrolysed so that after 24 hours only about 6-7% hydrolysis takes place. In contrast with this result, it may be stated that, according to work in progress in this laboratory (Jarvis, 1953), B.cinerea gives approximately 35% hydrolysis.

Conclusions.

The conclusions drawn from Figs. 7-11 and Table XXXVIII

may be summarised as follows:

(a) P.deBaryanum enzyme attacks pectin lowering its viscosity, but does not attack pectates, i.e. de-esterified pectin. (Fig. 7).

(b) P.deBaryanum enzyme causes very little change in the specific rotation of pectin solution (Fig. 10).

(c) P.deBaryanum enzyme, though reducing the viscosity of pectin solution, produces no breakdown products which can be recognised by the chromatographic method used (Fig.11).

(d) P.deBaryanum enzyme causes only 6-7% hydrolysis of pectin solutions in 24 hours when assayed by the reducing group method (Table XXXVIII).

(e) P.deBaryanum enzyme differs in most of these respects (a-d) from that of B.cinerea. The latter attacks pectates more rapidly than pectin (Fig. 8), causes marked decrease in the specific rotation of pectin solution (Fig. 10), attacks pectin to produce galacturonic acid and a series of poly-galacturonic acid compounds (Fig. 11) and causes much greater hydrolysis of pectin solutions. (Jarvis l.c.).

(f) P.deBaryanum enzyme shows some similarities with B.aroideae enzyme; both are incapable of causing significant change in specific rotation of pectin solution (Fig. 10) and in not producing products

products recognisable by chromatographic method used (Fig. 11) in contrast with B.cinerea.

From the above results it may be concluded that P.deBaryanum and B.aroideae do not secrete polygalacturonase in the conditions given. On the otherhand B.cinerea has got PG in its enzyme preparation.

In Fig. 12, the effects of P.deBaryanum enzyme on viscosity (V), specific rotation (S), and reducing capacity (R) of pectin solutions have been compared and show striking differences. The rapid and almost complete reduction in viscosity is associated with only small changes in specific rotation and reducing power of the solutions even after long reaction periods. Similar but less striking differences are also given by B.aroideae preparations. Both suggest the action of a 'depolymerase' type of enzyme.

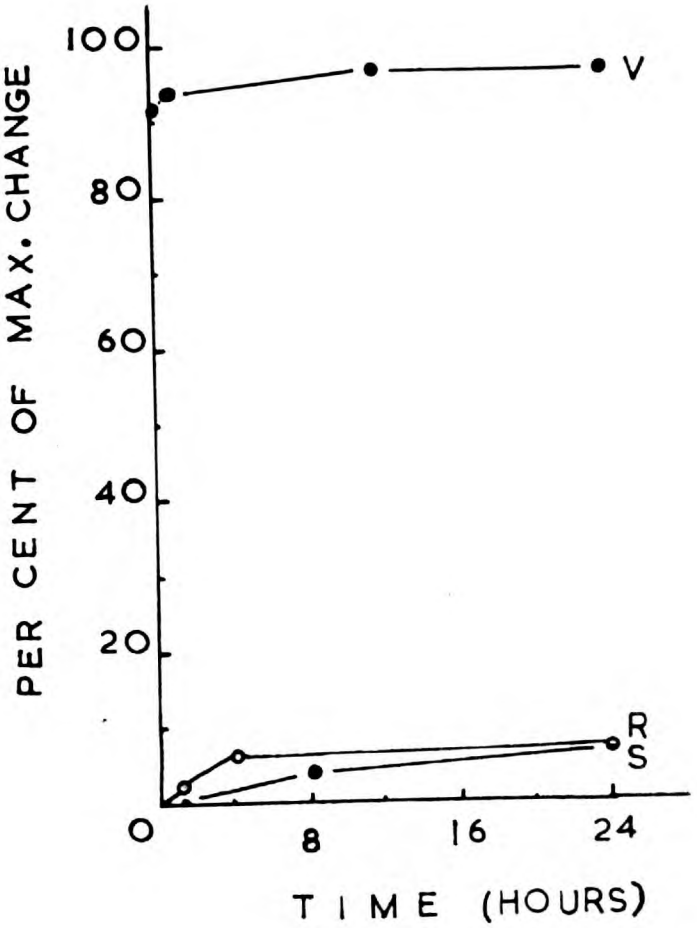

Fig. 12. Relationship between changes in viscosity (V), reducing groups (R), and specific rotation (S) of pectin solution caused by pectic enzymes of P.deBaryanum.

2. Physico-chemical Properties of Pectolytic Enzymes Secreted by Pythium deBaryanum.

Five factors have been examined in this section - temperature, concentration, pH, degree of purification, and thermal inactivation, and the results are set out in that order.

(a) Temperature.

The activity of the enzyme solution, secreted by P.deBaryanum, was tested at various temperatures by the maceration method. The results are illustrated in Fig. 13.

The activity of protopectinase ($\frac{100}{R.T.}$) increased by 100% between 10°and 20°C and by 50% between 20° and 30°C. No further increase was noted above 30°C presumably owing to the onset of enzyme inactivation at these temperatures. The enzyme (see below) has a very low temperature of inactivation.

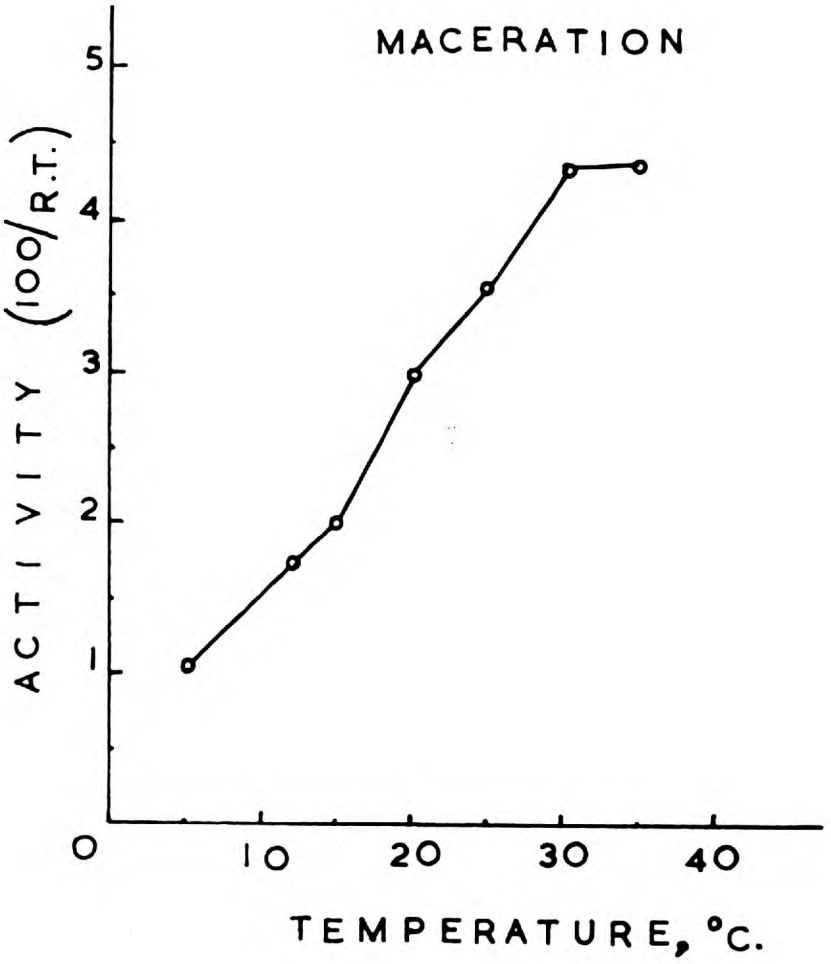

Fig. 13. Effect of temperature on rate of maceration by P.deBaryanum enzyme solutions.

(b) Dilution.

Various proportions of untreated and autoclaved enzyme solutions from P.deBaryanum cultures were mixed and the effect of dilution on activity studied by the maceration and viscosity methods.

Fig. 14 shows the relation between dilution and activity as represented by the value $\frac{100}{\text{R.T. (Mins.)}}$. Over the dilution range 10-100% the relation is approximately linear. Below a dilution of 10%, activity falls off more rapidly.

Fig. 15 records the effect of various concentrations of the enzyme solution on the rate of the viscosity loss of a 0.5% pectin solution.

If the activity of the full strength enzyme solution (100%) is considered to be 100 units, the corresponding activities for the various dilutions may be calculated in the following way. It is seen in Fig. 15 that the enzyme solution of 66.6% concentration requires 30 minutes to reduce the viscosity to the same point as is reached by the full concentration enzyme solution in 20 minutes (X, Fig. 15). The activity therefore of the 66.6% solution is $\frac{20}{30} \times 100$ = 66.6 units. The activity of other dilutions was similarly calculated. Fig. 16 shows that the relation between the concentration of enzyme and activity so calculated is linear.

(c) pH.

Measurments of the activities of enzyme preparations

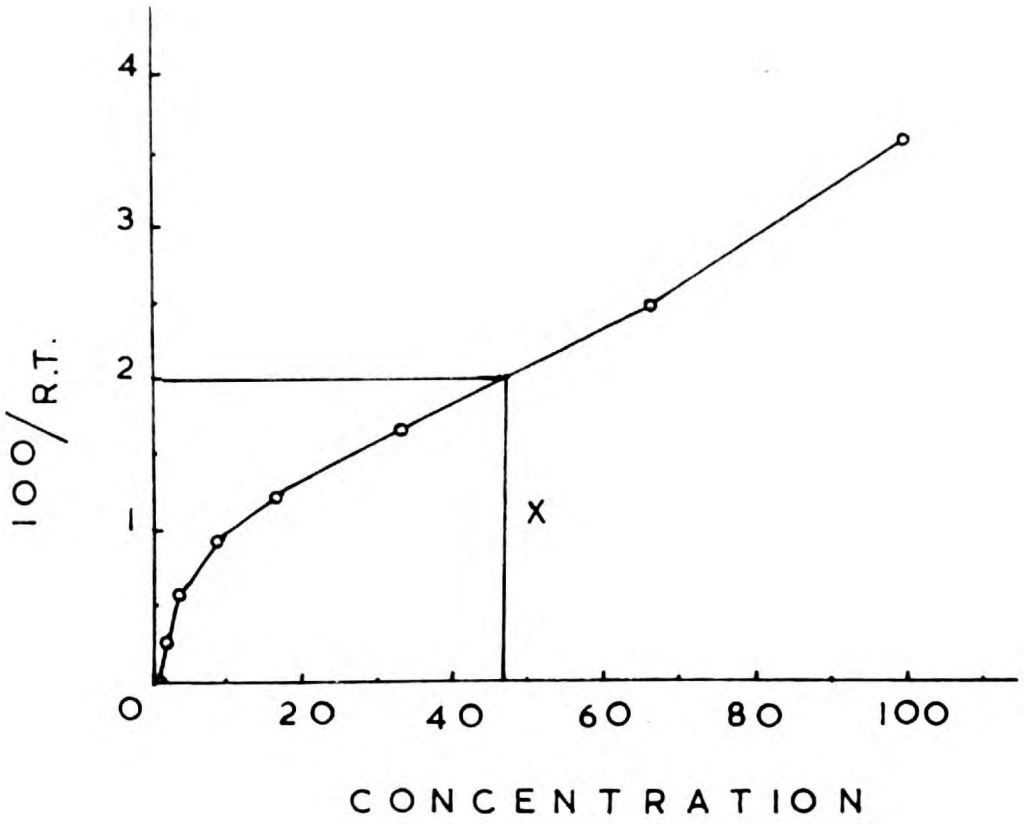

Fig. 14. Effect of concentration on maceration activity of P.deBaryanum enzyme.

X - Transformation of $\frac{R.T.}{100}$ value into enzyme units.

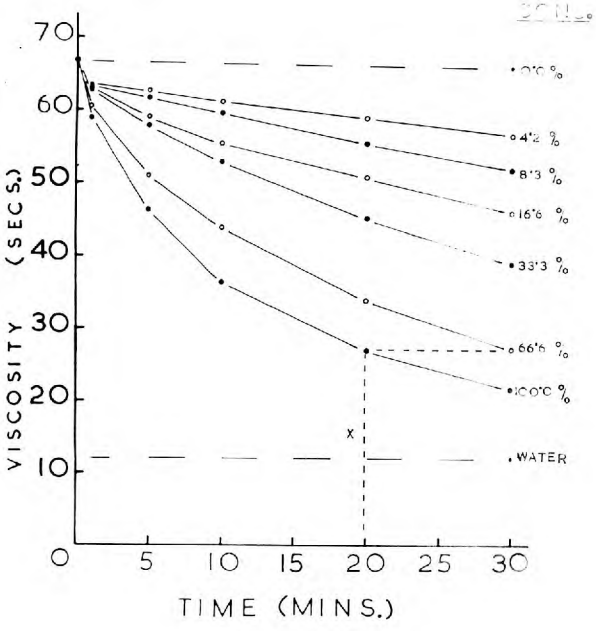

FIG. 15

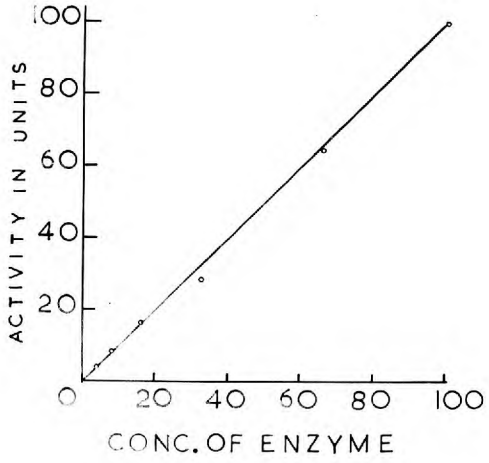

FIG. 16

Fig. 15. Effect of enzyme concentration (P.deBaryanum) on viscosity of pectin solutions.

Fig. 16. Relation between activity and enzyme concentration (P.deBaryanum) assayed by the viscosity method.

from P.deBaryanum cultures were made over a range of pH values by two methods:- (A) by maceration of potato discs, and (B) by viscometry.

The effect of pH on viscosity reducing activity of the enzyme solutions was plotted in a series of curves similar to those of Fig. 15, but the data were transformed into units of enzyme in the manner described for Fig. 16, the activity at pH 8.3 being considered as 100 units. The results are shown by curve B of Fig. 17.

The relationship of maceration to pH was also transformed by use of the dilution curve given in Fig. 14. At each pH value the figure $100/R.T.$ was transfered to the corresponding concentration, that of the enzyme at pH 8.3 being taken as 100. For example, a maceration activity ($\frac{100}{R.T.}$) of 2 represents a concentration unit of 46.5 (Fig. 14, X). The results are shown by curve A of Fig. 17.

Both curves agree in rising as the pH value goes from 5 upwards, i.e. both have optima at an alkaline pH. The response however is not the same in detail, as above pH 5.0 the rate of maceration increases more rapidly than the rate of viscosity loss. This might be taken as indicating that the macerating and viscosity attacking enzymes are not identical, but the evidence is not sufficient for such a conclusion since the direct effect of pH itself on cell wall substance is not known. There is the further consideration that the maceration

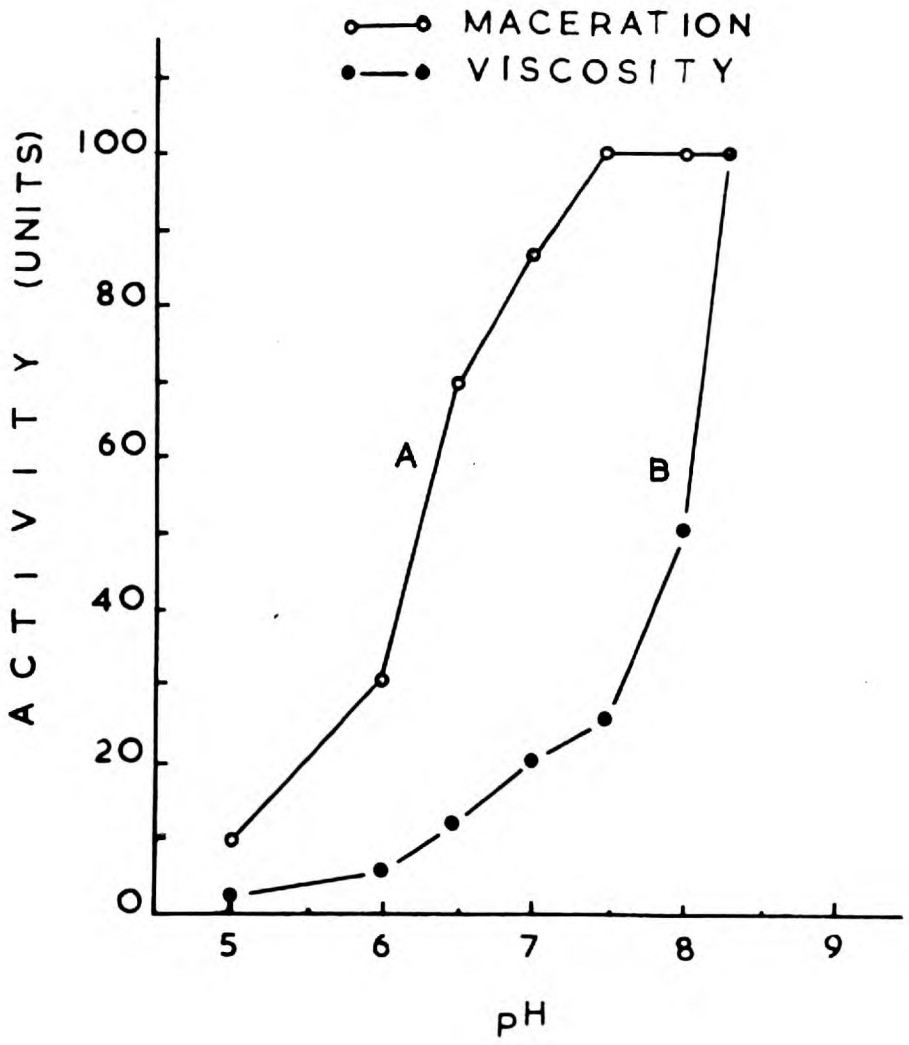

Fig. 17. Effect of pH on the activity of pectolytic enzymes of P.deBaryanum.

action takes place in a heterogeneous medium, so that purely physical factor like rate of diffusion may exert a controlling influence. It would therefore be unwarranted to conclude that the two enzymes are different on the basis of the small difference presented.

(d) Purification of Enzyme.

Purification of enzyme solutions obtained from liquid cultures of P.deBaryanum was carried out by one of two methods: (1) dialysis, and (2) precipitation.

Dialysis.

An enzyme solution was placed in a cellophane tube with a few drops of toluene. The open end of the bag was tied so as to leave no empty space, and then immersed in 600 mls. distilled water for 60 hours at 4°C. The water was changed every 10-12 hours.

After dialysis the pH of the solution had dropped from 8.2 to 5.8 and this was readjusted by adding 0.1 N NaOH. The activity of the dialysed enzyme solution with or without addition of salts, was tested, giving the results as shown in Figs. 18 and 19.

Dialysis greatly reduced the activity as tested either by the viscosity or the maceration method. The addition of magnesium sulphate or sodium chloride did not increase the activity, but the addition of phosphate resulted in some recovery of the lost activity. The addition of an autoclaved undialysed preparation had practically no effect.

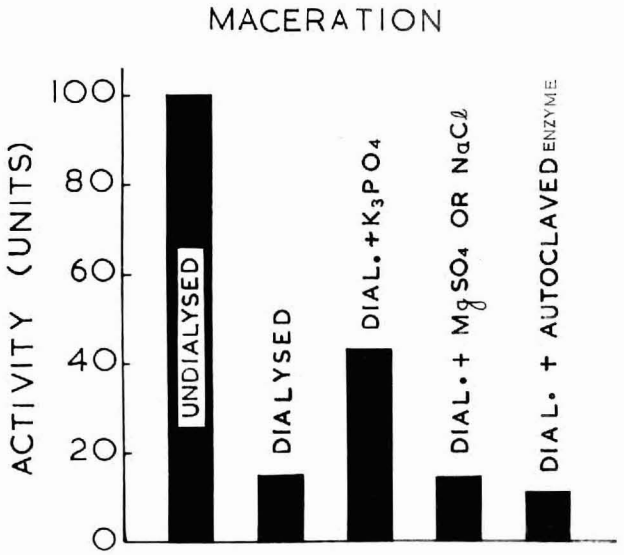

FIG. 18

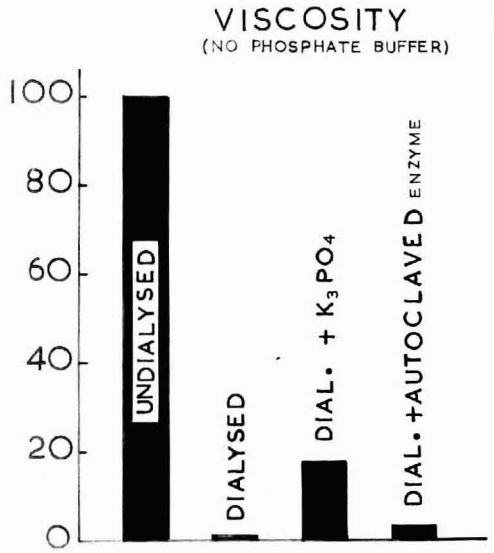

FIG. 19

Figs. 18 & 19. Effect of dialysis on the activity of pectic enzymes of P.deBaryanum.

Precipitation.

The precipitant alcohol or acetone, was added to enzyme solutions at pH 8.3 to the desired concentration and the mixtures left undisturbed at -20°C for three hours. The precipitate was then collected by centrifuging for 5 minutes at 2,500 r.p.m. Water, equal to the volume of the original enzyme solution, was used to redissolve the precipitate.

Fig. 20 records the activities of enzyme solutions after precipitating in 50, 60 and 75% acetone or alcohol.

It is seen that the enzymes responsible for maceration and loss of viscosity of pectin solutions, behave very similarly under these conditions. Recovery of enzyme after precipitating with 50 and 60% of the precipitant is very poor and the best (i.e. with 75% acetone) represents about half of the original activity.

Enzyme solutions were also precipitated over a range of pHs by using 60% acetone. Before mixing, enzyme solutions were adjusted to the required pH by addition of 0.1 N NaOH or HCl solution. Any change of pH caused by precipitation at the time of mixing was corrected by adding more NaOH or HCl solution. The precipitate was dissolved in the original volume of water and the pH readjusted to 8.3. The activity of enzyme recovered was tested by viscometry and the results are illustrated in Fig. 21. These show that precipitation at pH 8.3 which is also optimal for activity,

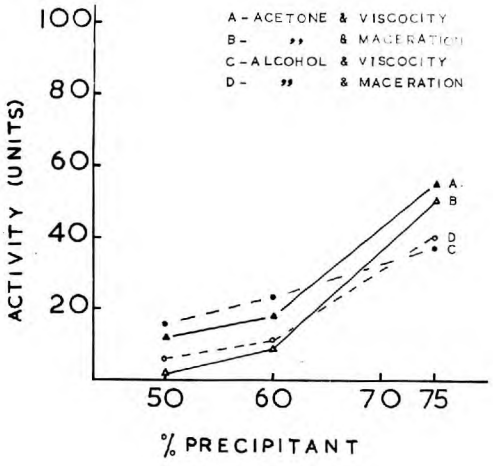

FIG. 20

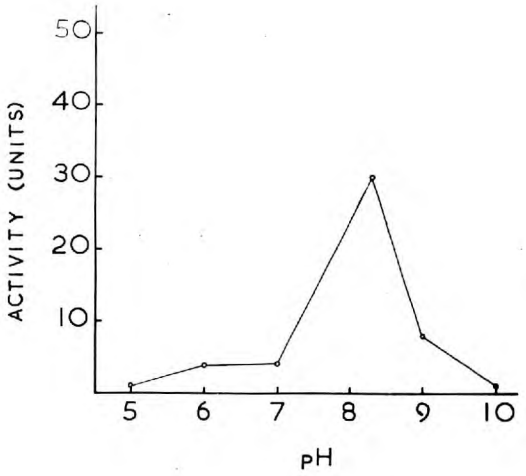

FIG. 21

Fig. 20. Effect of precipitation on the activity of pectic enzymes secreted by P.deBaryanum.

Fig. 21. Effect of precipitation, carried out at different pH values, on the activity of pectic enzyme s secreted by P.deBaryanum.
(activity assayed by viscometry only).

gave the best recovery.

In general it was concluded from Figs. 18-21 that purification, either by dialysis or by precipitation, of crude enzyme preparations of P.deBaryanum greatly reduced their activity.

(e) Thermal inactivation.

For tests, 5 mls. of the enzyme was placed in a thin walled glass tube which had been already warmed to the desired temperature. The tube was then held, with active stirring, in a water bath at the same temperature, and maintained there for a given time after the contents of the tube had reached the temperature of the water bath. It was then quickly cooled in ice-water. The activity of enzyme solutions, after such treatment, was measured by maceration and viscosity methods.

Fig. 22 shows the effect on macerating activity of P.deBaryanum enzyme after 1-30 mimutes exposure to temperatures over the range 35-55°C. Rapid inactivation of enzyme was noted at 45° and above, and was complete after one minute at 55°C. An exposure of 1 minute is not easy to carry out accurately; therefore the duration of exposure was lengthened to 5 mimutes in further experiments.

Fig. 23 gives the activity of P.deBaryanum enzyme after 5 mimutes exposure to a series of temperatures (25 - 100°C).

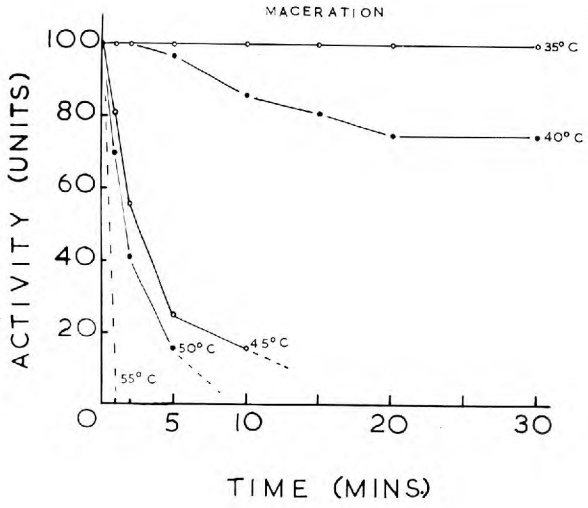

FIG. 22

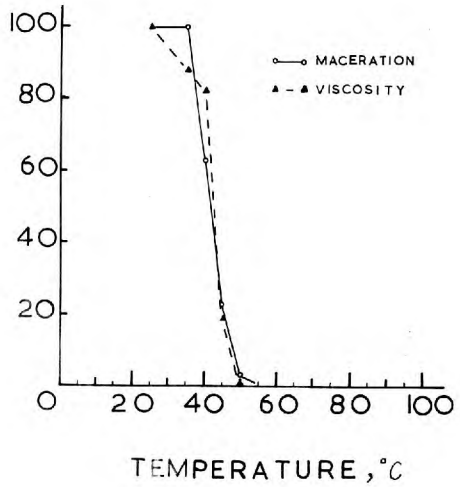

FIG. 23

Fig. 22. Isothermal inactivation of pectic enzymes secreted by P.deBaryanum.

Fig. 23. Thermal inactivation of pectic enzymes secreted by P.deBaryanum.

It is seen that the macerating or viscosity reducing activity of the enzyme, after treatment at 45°C, rapidly falls and disappears between 50°-55°C.

Figs. 24 and 25 record the corresponding behaviour of enzyme solutionsfrom cultures of B.cinerea and B.aroideae.

The difference in behaviour between B.cinerea and P.deBaryanum, if there is any, is very slight. Both are deactivated rapidly and completely at 50°-55°C. The enzyme of B.aroideae is more resistant; after 5 minutes' exposure at 65°C it still retains a trace of the viscosity-reducing enzyme. The macerating enzyme is even more resistant; it is reduced to a low level after 5 minutes' exposure at 75°C, but some activity is recorded on further heating and appreciable activity is shown even after 5 minutes' treatment at 100°C. Talboys (1950) records a similar result in his experiments. The persistance of a slow enzymic activity after exposing to temperatures above 70°C may be interpreted as meaning that B.aroideae pectolytic enzyme consists of two fractions, one which is the more active and is thermolabile, the other which is the weaker but is relatively thermostable. The low activity shown in Fig. 25 at 70°C. is more difficult to understand and was at first thought to be accidental. Repeats of the experiment, however, have given fuller evidence of the existence of this peculiar effect, as shown in Table XXXIX.

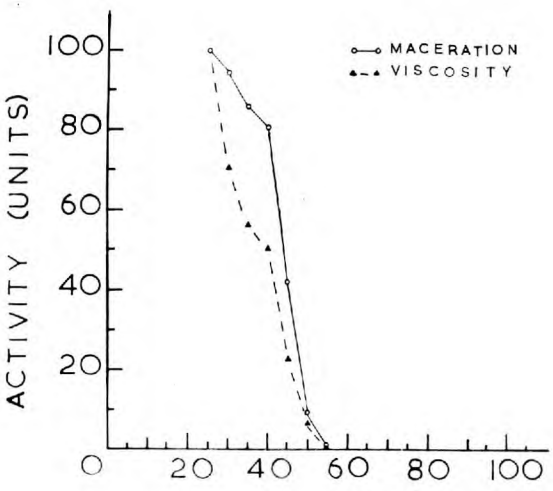

FIG. 24

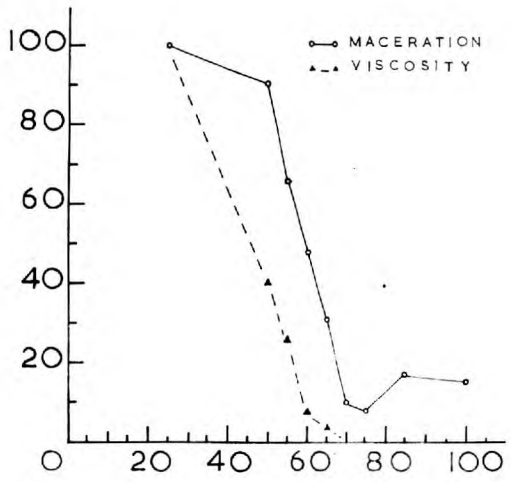

FIG. 25

Fig. 24. Thermal inactivation of pectic enzymes secreted by B.cinerea.

Fig. 25. Thermal inactivation of Pectic enzymes secreted by B.arcideae.

Table XXXIX.

Effect of Heating on Macerating Activity of B.aroideae enzyme.

Source of enzyme	R.T. (Mins.) of untreated enzyme	R.T. (Mins.) after treating the enzyme for 5 minutes at temps.		
		70°C	85°C	100°C
1. Crude, from cultures.	25	140	85	80
2. As above, but concentrated by precipitation.	10	4 hrs.	4 hrs.	3 hrs.

In this experiment the effect is most clearly shown by the crude enzyme preparation; it is only doubtfully shown by the concentrated enzyme. The same kind of result, with B.aroideae enzyme, has been experienced by other workers in this laboratory.

II DISCUSSION

This may conveniently be divided into two parts in conformity with the general arrangement of the experimental work.

First Part.

In general the findings of Ashour (1949), that Pythium deBaryanum is rather selective in its requirements for free pectinase secretion, were confirmed. Any variation in constituent or in concentration, from that devised by Ashour in his synthetic medium, led into reduced enzyme production. The main improvement introduced in this work has been the addition of a small quantity of sodium chloride, which further stimulates enzyme production. Ashour's medium was made up without such addition and as he reports active secretion of enzyme, it could be assumed that some constituent of his medium was responsible for introducing the salt as an impurity. That certain ions such as $Cl^{'}$ favour enzyme secretion and others such as $Ca^{''}$ have the opposite effect is a result which has come out of the present work.

Damle's (1952) interesting finding, that pectinase is not freely secreted by P.deBaryanum unless the carbohydrate (glucose) constituent is autoclaved with phosphate, has also been confirmed. Whereas, however, he suggests that this beneficial effect may be due to the formation of a phosphoric

ester of glucose or fructose, the present work with the help of chromatographic analysis indicates that such phosphate is not formed. The beneficial effect given by dry heated glucose (caramel), or by autoclaving glucose in alkaline solution (NaOH), suggests that heating of this sugar with potassium phosphate (tribasic) only brings about the caramelization of glucose due to its alkaline reaction. These results recall the earlier work of Fulmer & Huesselmann (1927), Fulmer et al (1931), Lewis (1930), and Baumgartner (1938) already cited (pages 6 & 7). With the help of chromatographic method, the writer has shown that glyceraldehyde is formed during caramelization of glucose and that it has got stimulating effect on enzyme production of P.deBaryanum.

The effects of various carbohydrates on enzyme secretion, as worked out in this paper, agree in the main with Ashour's and Damle's results, but there are some divergences as well. There is general agreement that sucrose alone as source of carbon is inferior to a mixture of starch and glucose. In the writer's experience, however, sucrose when accompanied by a small amount of glucose is as suitable as starch ~~and~~ with glucose, a conclusion which is in disagreement with Ashour. In view of the effect of glyceraldehyde, which has just been described and which is formed on heating glucose and not sucrose, Ashour's results are unexpected and a mistake on his

part seems likely.

Three hexoses - glucose, fructose and mannose - have been found to be suitable for enzyme production by P.deBaryanum. All these three sugars give the same enolic form with hydroxyl migration, and this property may be the reason for their equivalence as food substance for the fungus. On the otherhand galactose (also hexose) has a different configuration of the atomic groups, which may be the reason why it is quite unsuitable for growth and enzyme production by P.deBaryanum.

Second Part.

The most interesting results presented in this part relate to the nature of pectolytic enzymes secreted by Pythium deBaryanum. When Pythium enzyme is tested upon pectic substances it readily reduces the viscosity of pectin solutions but has no effect on pectate solutions. In its action on pectin it gives no (mono-) galacturonic acid or any intermediate degradation product which can be detected by the chromatographic method. Its effect on optical rotation of pectin solution and on the production of reducing groups is negligible. There is therefore no polygalacturonase present in pectolytic enzymes secreted by P.deBaryanum, on the other hand it has got an enzyme of 'depolymerase' nature - a type which has previously been reported by Beaven & Brown (1949), Wood (1951), Seegmiller

& Jansen (1952), and Roboz, Barrat & Tatum (1952). By the last mentioned workers (Roboz et al) this enzyme has been named as Pectin-depolymerase. The detailed properties of the enzymes described by these workers are not in full agreement, but all agree in the main property of the enzyme of reducing the viscosity of pectin solutions without production of detectable galacturonic acid. Pythium enzyme shows more similarity with the enzymes described by Beaven & Brown (l.c.) and Seegmiller & Jansen (l.c.).

This work on pectolytic enzymes secreted by P.deBaryanum supports the view of Kertesz (1939) that the high viscosity of pectic solutions is not due to large chains of anhydrogalacturonic acid units, but to aggregates consisting of these polyuronic acid units joined by linkages which are not glycosidic in nature. There is evidence in the literature (Jansen & MacDonnell, 1945; Matus, 1948; and Reid, 1951) that there are two phases in hydrolysis of pectic compounds: the first of disaggregation (by depolymerase) and the second of decomposition of polygalacturonic acid units (by polygalacturonase).

The absence of polygalacturonase from enzyme preparation of P.deBaryanum raises doubts about the view that protopectinase and polygalacturonase are the same enzyme (Kertesz, 1951; P. 335). The main evidence in its favour has been the presence of polygalacturonase in all

protopectinase preparations so far examined. The existance of a depolymerase type of enzyme is a later discovery and it is obvious that when polygalacturonase is present, the existance of depolymerase enzyme would be masked. In view, therefore, of enzymic system of P.deBaryanum, which contains a protopectinase and depolymerase but no polygalacturonase or pectin-esterase, it is more appropriate to suggest that polygalacturonase is a separate enzyme but protopectinase may be identical with depolymerase. More work of this kind, with a variety of micro-organisms, will probably lead to a better understanding of the structure of pectic substances and nature of their enzymes, together with the precise manner in which cell wall tissue is hydrolysed by a parasite.

III SUMMARY.

1. Potato extract medium prepared in three ways, was found to be satisfactory for enzyme secretion by Pythium deBaryanum.

2. The incorporation of sodium chloride in a synthetic medium (Ashour's Medium) stimulated enzyme secretion. The $Cl^{'}$ ion was especially important in this connection. Presence of $Ca^{''}$ ion on the otherhand depressed enzyme production.

3. Addition of pectin to Ashour's Medium also stimulated production of pectolytic enzymes, but gave no improvement when added to former medium which already contained sodium chloride. Pectin, as sole source of carbon in the medium, gave poor growth of the fungus and enzyme production.

4. No improvement in enzyme production was obtained by adding certain growth substances - biotin, aneurin, nicotinamide and riboflavine - to Ashour's Medium.

5. Replacement of either the asparagine or the peptone of the standard medium by nitrates or by ammonium salts depressed enzyme production.

6. Various carbohydrates were compared; glucose, fructose, and mannose were found to be suitable; sucrose or starch was poor if used alone but gave good results if a small proportion of glucose was present; galactose was not utilised by P.deBaryanum.

7. For optimum enzyme production glucose (or fructose or mannose) is required to be autoclaved with potassium phosphate (either neutral or alkaline). This method of preparing the medium led to the formation of a brownish colour (due to caramelization) but the stimulating factor was not bound up with the colouring substance. The same stimulating substance was obtained by adding to the synthetic medium a small quantity of glucose which had been dry heated at 150°C for 20 minutes. Chromatographic analysis suggested that the stimulating substance was probably glyceraldehyde.

8. Pectinesterase is not present in the enzyme system of P.deBaryanum. On the otherhand tissue macerating enzyme - protopectinase - and pectin viscosity reducing enzyme are present. Pectic acid solutions are not affected.

9. Though the viscosity of pectin solutions is readily reduced by Pythium enzyme, it is unable to cause substantial hydrolysis of pectin to produce (mono-) galacturonic acid

or detectable intermediate products; this fact is supported by the little change in reducing capcity and optical rotation of pectin solution.

10. In accordance with present definitions, poly-galacturonase is absent from pectolytic enzymes secreted by P.deBaryanum. The viscosity reducing property is due to another enzyme which may be called as Pectin-depolymerase.

11. The protopectinase and pectin-depolymerase of P.deBaryanum have their optima at about pH8, and both are deactivated by heating to about 50°C for 5 minutes.

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